ANALYSING SINGLE NUCLEOTIDE VARIATION IN DRUG RESISTANT STRAINS OF *Mycobacterium tuberculosis* USING BOWTIE & SAMTOOLS

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CERTIFICATE

This is to certify that the M. Tech. dissertation entitled **"ANALYSING SINGLE NUCLEOTIDE VARIATION IN DRUG RESISTANT STRAINS OF *Mycobacterium tuberculosis* USING BOWTIE & SAMTOOLS"** submitted by **ANU KHERA (2K11/BIO/02)** in partial fulfilment of the requirement for the award of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance.

The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honouring of any other degree.

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DECLARATION

I hereby declare that this project titled "**ANALYSING SINGLE NUCLEOTIDE VARIATION IN DRUG RESISTANT STRAINS OF *Mycobacterium tuberculosis* USING BOWTIE & SAM TOOLS**", submitted to Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), Delhi is the original and independent work carried out by me under the guidance of Dr. Jai Gopal (Associate Professor), Department of Biotechnology, Delhi Technological University, New Delhi, in partial fulfilment of the requirements for the award of Degree of Master of Technology in Bioinformatics and this dissertation or part of it has not been submitted by me for any other degree /diploma in any University/ Institute.

Anu Khera

2K11/BIO/02

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Anu Khera
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LIST OF ABBREVIATIONS

AIDS	Acquired Immuno Deficiency Syndrome
AFLP	Amplified fragment length Polymorphism
BAM	Binary Alignment/Map
BCF	Binary Call Format
BCG	Bacille Calmette Guerin
BWT	Burrows Wheeler Transform
DNA	Deoxyribonucleic Acid
DR	Drug Resistant
EAI	East African Indian
EMBL	European Molecular Biology laboratory
dN	Rate of non-synonymous substitutions
dS	Rate of synonymous substitutions
HGT	Horizontal Gene Transfer
HIV	Human Immuno deficiency Virus
IGRA	Interferon Gamma Release Assays
MDR	Multi Drug Resistant
MTBC	*Mycobacterium tuberculosis* Complex
NIH	National Institute of Health
qname	Variable used in SAM format
RNA	Ribonucleic Acid

SAM	Sequence Alignment/Map
SNVs	Single Nucleotide Variations
sSNVs SNVs	Synonymous SNVs
nsSNVs SNVs	Non-synonymous SNVs.
TB	Tuberculosis
Ts	Transitions
Tv	Transversions
VCF	Variant Call Format
WHO	World Health Organization
XDR	Extremely Drug Resistant
TB	Tuberculosis
Ts	Transitions
Tv	Transversions
WHO	World Health Organization

Analysing Single Nucleotide Variation in Drug resistant strains of *Mycobacterium tuberculosis* using BOWTIE & SAMTOOLS

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ABSTRACT

The occurrence of drug resistance in *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), is hampering the management and control of TB in the world. Genome-wide scans for genetic variation have been conducted with the aim of clarifying the genetic basis of disease, and recent advancements in DNA genotyping technologies have revealed many susceptibility genes and variants for common diseases. It examines the variations among the different strains of same species and relate with trait. The Single Nucleotide Variation in different strains of *Mycobacterium tuberculosis* were identified using BOWTIE & SAMTools and then examines the impact of SNVs on the virulence and pathogenicity. For this analysis, genome sequences of three strains of *Mycobacterium tuberculosis*: AAKR, AMXW and AQQC were analyzed for the single nucleotide variations with reference to the H37Rv, a pathogenic laboratory strain. The SNVs were found more in the genic region than in intergenic region. Transition to transversion ratios (Ts/Tv ratio) were also calculated in each strain and analysis of SNVs supports the transitional bias in all the strains. Then further categories the SNVs in different functional categories of TubercuList under lipid metabolism, information pathways, cell wall and cell processes, stable RNAs, insertion seqs and phages. PE/PPE, intermediary metabolism and respiration, regulatory protein, conserved hypotheticals etc. The results suggest that the drug resistant phenotype may be due to changes in a number of genes. This result will further give light on the evolution of drug resistant strains.

INTRODUCTION

According to World Health Organization (WHO) factsheet of March 2012, Tuberculosis (TB) is second only to HIV/AIDS as the greatest killer worldwide due to a single infectious agent. In 2011, 8.7 million people fell ill with TB and 1.4 million died from TB. It is caused by various strains of *Mycobacterium tuberculosis*, a successful pathogen that has latently infected one third of world population. Though, BCG vaccines are developed for the treatment of the disease, the worldwide emergence of drug resistant strains hampered progress in treatment and control of the disease. These organisms are also resistant to multiple drugs which limit the choice of drugs for TB control (Lawn and Wilkinson, 2006).

A number of studies are ongoing particularly in Mycobacteria to understand the genetic alterations in order to identify the mechanism involved in the resistance (Telenti 1998). These studies based on few target gene sequences. The other major drug resistance in M. tuberculosis is detection of polymorphisms based on methods such as spoligotyping (Sola *et al*. 2000), amplified fragment length polymorphism (AFLP) (Vosa *et al*. 1995) etc.

Availability of complete genome sequences of different strains of drug resistant Mycobacteria gives a way to understand genetic alteration and drug resistance. The comparison of genomic DNA sequence of different individuals (strains) revealed some variations like single nucleotide variations (SNVs), deletions and insertions. Some of the genetic differences, however, have proven to be very important in the study of phenotypic differences among drug resistant strains.

Single nucleotide variations are DNA sequence variations that occur when a single nucleotide (A,T,C,or G) in the genome sequence is altered. SNV might change the DNA sequence. These are the most common form of intra-species variations (Durbin *et al*, 2010). Single-nucleotide polymorphisms may fall within coding sequences of genes**,** non-coding regions of genes, or in the intergenic regions (regions between genes). Depending on where SNV occurs, it might have different consequences at the phenotypic level (Nicol and Wilkinson, 2008). The researchers divided the SNP data into two major classes: genic and nongenic. Further, the genic SNVs are differentiated into two catagories: synonymous (s SNVS) and non-synonymous (ns SNVs). Synonymous SNVs do not alter the amino acid sequence, whereas, non-synonymous SNVs change the amino acid sequence of a protein. Selection pressure would be stronger in non synonymous mutations of the genic region. (Barreiro *et al* 2008).

The analysis of dN/dS ratio, which is the most widely used statistical test, shows selective pressure in the protein coding region of the genome (Kryazhimskiy and Plotkin, 2008). dN/dS ratio refers to the number of non-synonymous substitution per non-synonymous site to the number of synonymous substitutions per synonymous site.

dN/dS >1 refers to the positive or purifying selection (changes allowed during natural selection), dN/dS<1 refers to purifying selection and dN/dS=1 refers to neutral selection or no selection (Yang *et al*, 2000). Therefore, SNVs may play more direct role in disease by affecting the function of a gene.

M. tuberculosis H37Rv is a pathogenic laboratory strain, derived from its parent H37, a clinical isolate obtained from a patient with chronic pulmonary tuberculosis (Zheng *et al*, 2008), and is most widely used strain for tuberculosis research. Genomes of three drug resistant strains AQQC, AAKR, AMXW analyzed for the single nucleotide variations with reference to H37Rv strain. These SNVs were then classified and analyzed for each functional category of TubercuList. Bowtie 0.12.7 (http://Bowtie.cbcb.umd.edu/; Langmead *et al*, 2009) is used for alignment of the strains and all the scripts for genome analysis and SNV detection written in R language using various packages of Bioconductor.

The combination of genomics and bioinformatics has the potential to generate the information and knowledge that will enable the conception and development of new therapies and interventions needed to treat this airborne disease and to elucidate the unusual biology of its etiological agent, *Mycobacterium tuberculosis*.

REVIEW OF LITERATURE

3.1. Tuberculosis

Tuberculosis (TB) is an infectious disease that is caused by a bacterium called *Mycobacterium tuberculosis*. TB is a major cause of illness and death worldwide, especially in Africa and Asia. Each year the disease kills almost 2 million people. The disease is also prevalent among people with HIV/AIDS.

It is transmitted by aerosols containing infectious *Mycobacterium tuberculosis* released from the lungs of infected individuals upon coughing. TB primarily affects the lungs, but it can also affect organs in the central nervous system, lymphatic system, and circulatory system among others *Mycobacterium tuberculosis*

3.1.1. History

Archaeological evidence from Egyptian mummies indicated manifestation of TB as early as 5000 BC (Schaaf & Zumla 2009). In the 18th and 19th centuries, TB reached epidemic proportions in Europe and North America and was referred to as “The Great White Plague” (Dubos 1952). On March 24, 1882, the German scientist Robert Koch changed the course of TB history when he identified *Mycobacterium tuberculosis* as the causative agent of TB. The discovery of the tubercle bacillus paved the way for great advances in the areas of TB diagnosis, prevention and control.

3.1.2. Epidemiology

According to the Global Tuberculosis Report 2012, tuberculosis remains to be problem worldwide, estimates that there were 8.7 million new cases in 2011 and 1.4 million deaths. In 2010 there were an estimated 8.8 million cases of TB and 1.4 million estimated deaths caused by TB. The highest incidence rate was observed in Asia and African low and middle income counties (Figure 1), reflecting the high prevalence of HIV infection in this region (Raviglione *et al*. 2012). Over 80% of the worldwide TB burden is borne by 22 low-income and middle income countries, with the majority of the estimated cases occurring in Asia (59%) and Africa (26%). The three countries with largest number of incident cases in 2010 were India, China, South Africa, Indonesia, and Pakistan (World Health Organisation, 2011b)

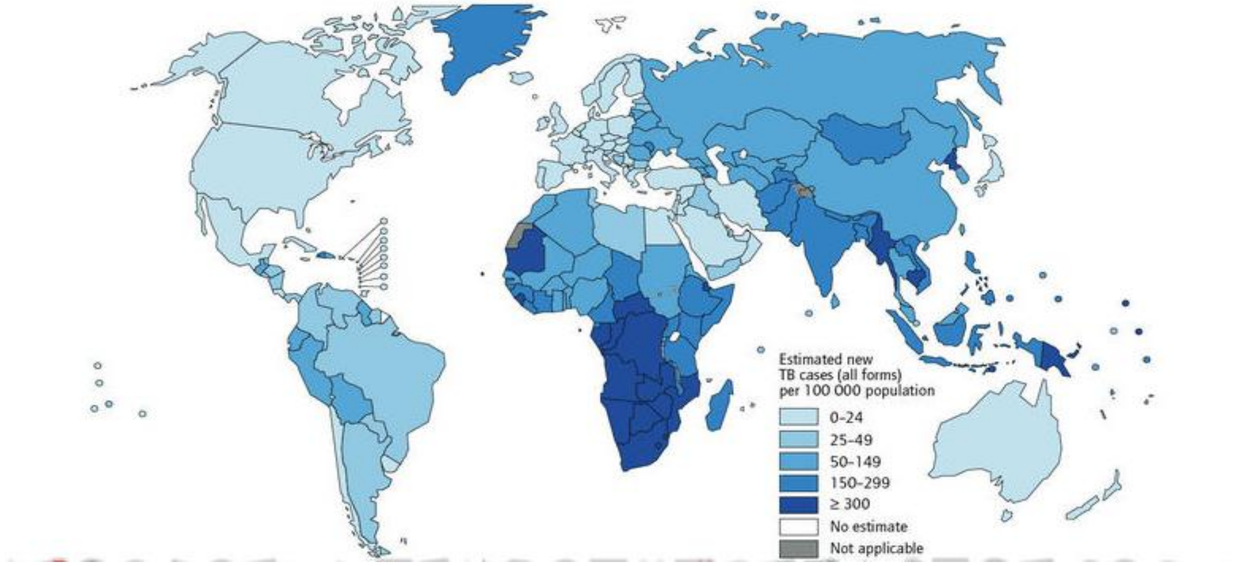


Figure1. Estimated tuberculosis incidence in 2011 (Global Tuberculosis Report, WHO, 2011b).

3.1.3. Symptoms

When a person becomes infected with tuberculosis, the bacteria in the lungs multiply and cause pneumonia along with chest pain, coughing up blood, and a prolonged cough. In addition, lymph nodes near the heart and lungs become enlarged. As the TB tries to spread to other parts of the body, it is often interrupted by the body's immune system. The immune system forms scar tissue or fibrosis around the TB bacteria, and this helps fight the infection and prevents the disease from spreading throughout the body and to other people. If the body's immune system is unable to fight TB or if the bacteria breaks through the scar tissue, the disease returns to an active state with pneumonia and damage to kidneys, bones, and the meninges that line the spinal cord and brain (Figure 2).

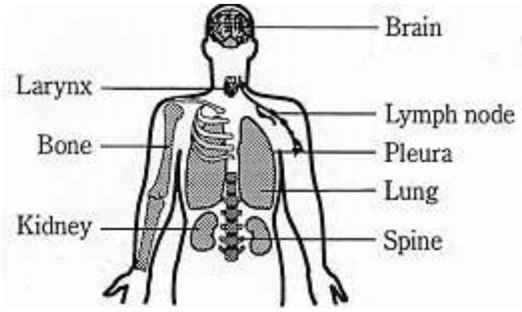


Figure 2. Common sites of Tuberculosis infection

3.1.4. Mechanism

TB infection begins when the Mycobacteria reach the pulmonary alveoli, where they invade and replicate within endosomes of alveolar macrophages (Houben *et al*, 2006). The primary site of infection in the lungs, known as the "Ghon focus", is generally located in either the upper part of the lower lobe, or the lower part of the upper lobe. Tuberculosis of the lungs may also occur via infection from the blood stream. This is known as a Simon focus and is typically found in the top of the lung (Khan, 2011). This hematogenous transmission can also spread infection to more distant sites, such as peripheral lymph nodes, the kidneys, the brain, and the bones.

3.1.5. Diagnosis

A diagnosis of TB when there is sign of lung disease or constitutional symptoms lasting longer than two weeks (Escalante *et al*, 2006). In the initial evaluation chest X-ray and sputum culture can be done. Tuberculin test now a days are of little (Metcalfe *et al* 2011). IGRA have similar limitations in those with HIV. A definitive diagnosis of TB is made by identifying *M. tuberculosis* in a clinical sample (e.g. sputum, pus, or a tissue biopsy). However, the difficult culture process for this slow-growing organism can take two to six weeks for blood or sputum culture. Thus, treatment is often begun before cultures are confirmed (NIH and clinical excellence guideline).

The Mantoux tuberculin skin test is often used to screen people at high risk for TB. Those who have been previously immunized may have a false-positive test result. (Rothel *et al*, 2005). Interferon gamma release assays (IGRAs), on a blood sample, are recommended in those who are positive to the Mantoux test.

3.1.6. Prevention , Treatment and limitation

The DOTS program treatment involves administration of four drugs isoniazid, rifampicin, pyrazinamide and ethambutol for the first two months followed by four months of isoniazid and rifampicin. After 2007, there is lots of tuberculosis strain that are choice resistance to drugs. Hence, there is an urgent need to develop TB regimens that can cure all forms of TB and that are safe and compatible.

Since 1921, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) has been the only vaccine used against tuberculosis. It is a live attenuated vaccine that was derived upon 230 repeated subcultures of a strain of *Mycobacterium bovis* on potato slices soaked in glycerol and ox bile, leading to the in vitro accumulation of mutations and ultimately attenuation (Calmette 1931). Over 4 billion people have been vaccinated with BCG so far making it the most widely used vaccine in the world (Kaufmann & Hussey 2010). BCG is effective against severe forms of TB in children (Trunz *et al*. 2006), but it offers very little protection against adult pulmonary TB, the most prevalent form of the disease (Fine 1995). In addition, BCG is not recommended for use children diagnosed as HIV-positive owing to the risk of disseminated BCG.

3.2. *Mycobacterium tuberculosis*

M. tuberculosis is a pathogenic, gram-positive bacterial species of genus. Based on the evidence accumulated from these studies, it has been suggested that *M. tuberculosis* evolved in Africa as a human pathogen, where all main M. tuberculosis lineages have been isolated (Hershberg *et al*, 2008). It belongs to the *Mycobacterium tuberculosis* Complex (MTBC), a group of slow growing Mycobacteria that are closely related at the DNA level but their phenotypes and host preferences are different (Brosch *et al*, 2001; Sreevatsan *et al*, 1997). The MTBC includes the human-adapted strains *M. tuberculosis*, *M. africanum* and *M. Canneti*, being *M. cannetti* the most divergent within the MTBC complex (Gutierrez *et al*, 2005).

M. tuberculosis H37Rv is a pathogenic laboratory strain, derived from its parent H37, a clinical isolate obtained from a patient with chronic pulmonary tuberculosis (Zheng *et al*, 2008), and is most widely used strain for tuberculosis research. The complete genome sequence and annotation of this strain was published in 1998 (Cole *et al*, 1998) (figure 3). The information from this project was incorporated into the public database TubercuList (http://tuberculist.epfl.ch/). The circular *M. tuberculosis* H37Rv genome consists of 4,411,532 bp and displays a high G+C content of 65% (Table 1). It contains over 4000 protein-coding and 50 stable RNA genes, using around 91% of its potential coding capacity. It displays a gene density at one gene per kilobase, which is comparable to other prokaryotes. Genes are evenly distributed on the forward and reverse strands, and 59% of the transcription is in the same polarity as the replication fork. More than half of the coding sequences in *M. tuberculosis* have arisen from gene duplication or domain-shuffling events (Tekaia *et al*. 1999).

DNA sequence	4,411,532 bp
Protein genes	4,018
Pseudogenes	13
tRNA genes	45
rRNA genes	3
Nc RNA genes	30
Misc RNA genes	2
GC percentage	65.9 %
Gene density	0.91 genes per kb
Average length	1,002 bases per gene
Protein coding percentage	91.2 %
Protein coding bases	4,027,296

Table 1: Genomic features of *Mycobacterium tuberculosis* H37Rv strain.

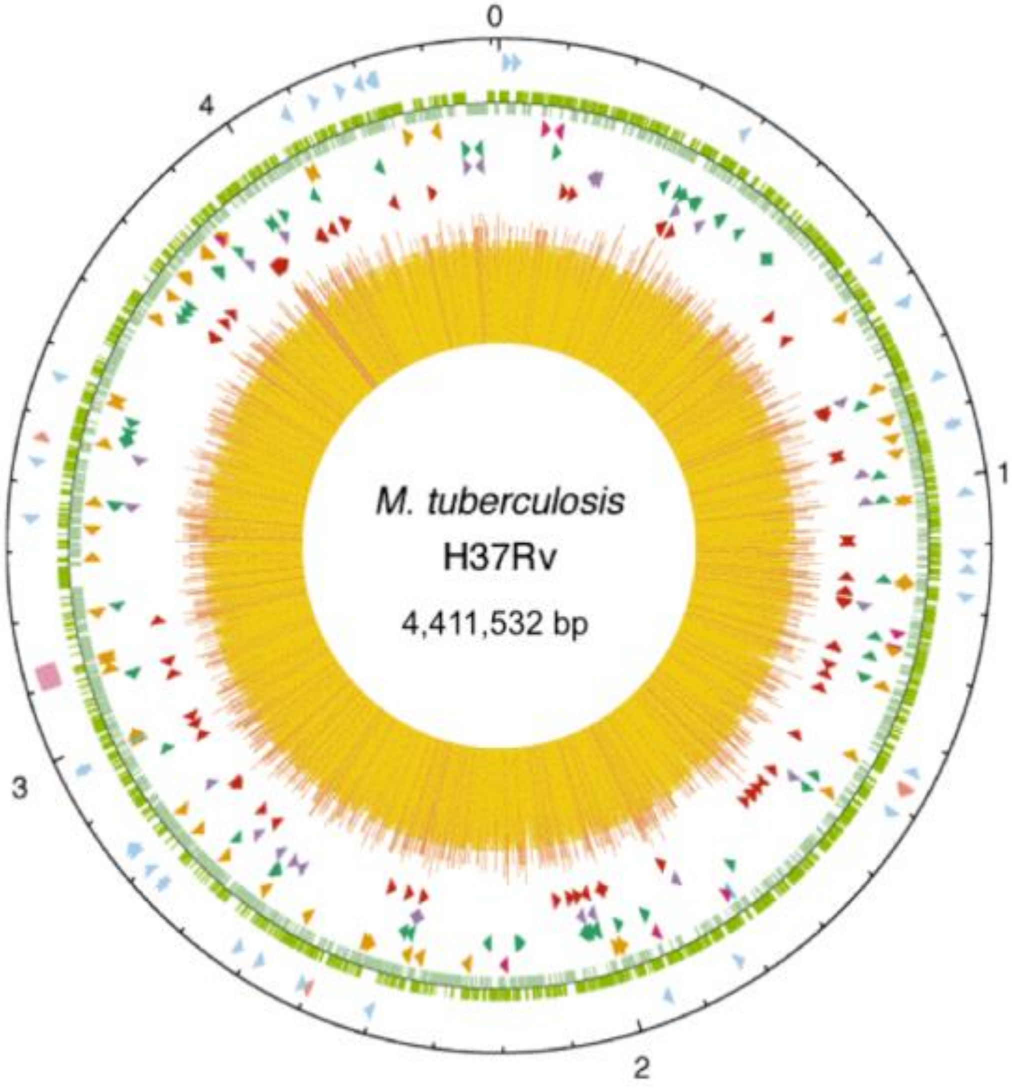


Fig 3. Circular map of the chromosome of *M. tuberculosis* H37Rv (S. Cole *et al*. 1998). *The outer circle shows the scale in megabases, with 0 representing the origin of replication.* *The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, and others are pink) and the direct-repeat region (pink cube); the second ring shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the positions of the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (center) represents the G+C content, with <65% G+C in yellow and >65% G+C in red (S. Cole et al. 1998).*

The *M. tuberculosis* genes have been broadly classified into eleven functional categories (Table 2). The genome sequence highlighted several characteristics of the biology of *Mycobacterium tuberculosis*, which have been summarized below for each functional category.

Category	Function	Number of features	% of total
0	Virulence, Detoxification and adaptation	238	5.8
1	Lipid metabolism	272	6.6
2	Cell wall and cell processes	773	18.9
3	Stable RNAs	73	1.8
4	Insertion seqs and phages	147	3.6
5	PE/PPE proteins	168	4.1
6	Intermediary metabolism and respiration	936	22.9
7	Unknown	16	0.4
8	Information pathways	242	5.9
9	Regulatory proteins	198	4.8
10	Conserved hypotheticals	1032	25.2

Table 2. Broad classification of M. tuberculosis genes according to the functional categories of TubercuList database (Lew *et al*. 2011).

3.3. Drug resistant *Mycobacterium tuberculosis* strain

As of August 2012, the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the European Bioinformatics Institute (http://www.ebi.ac.uk/) databases hold complete genome sequences for 48 Mycobacterial strains and partial sequences (scaffolds/contigs) for 33 strains.

Discrimination between strains of pathogenic bacteria is crucial from an epidemiological perspective. The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis*is a major public health problem that threatens tuberculosis (TB) care and control in many countries. Molecular ‘strain typing’ or genotyping tools have been used to study disease outbreaks and transmission (Small *et al*. 1994), to assess the relative contributions of reinfection and reactivation to disease (Van Rie *et al*. 1999), and to address a number of other issues relevant to TB control.

Though, tuberculosis is curable yet it has become one of the leading diseases due to the development of drug resistant strains. These strains hurdles the cure of this disease and make it untreatable. The cause of emergence of these multi drug resistant strains was assumed to be the reduced purifying selection constraints. This reduced constraint might allow the mutation to persist in the drug resistant strain even in the absence of the antibiotic. Therefore this persistence of mutation might have role in the epidemic emergence of the multidrug resistant strains of

M. tuberculosis and so multidrug resistant tuberculosis (Hershberg *et al*, 2008).

The drug resistant strains AQQC, AMXW and AAKR isolated from different countries match with the reference genome H37Rv in order to show the variations. AQQC, AMXW, and AAKR are the whole genome shotgun project.

AAKR, AMXW and AQQC consist of whole sequence contigs of *Mycobacterium tuberculosis* strain C, X122 and EAI/OSDD271 resp. AAKR is a drug-susceptible strain that has caused a large proportion of new tuberculosis cases in New York. AAMX is the first isolate of Extensively Drug-Resistant (XDR) *Mycobacterium tuberculosis* in Malaysia.

AQQC is being recently sequenced by IGIB Institute, Delhi is Multidrug resistant *Mycobacterium tuberculosis* East African Indian (EAI) clinical isolate OSDD271.

3.3.1. R and Bioconductor:

R (http://www.r-project.org/) language is a project designed to create a free, open source language, initially written by Ross Ihaka and Robert Gentleman at the Department of Statistics of the University of Auckland in Auckland, New Zealand. It provides statistical environment and programming language for data analysis and graphics. Bulk of data is generated nowadays using high throughput techniques. Thus, we need to develop the algorithms to perform fast on such big data. R is a complete, interactive, object-oriented language: designed by statisticians, for statisticians. The language provides objects, operators and functions that make the process of exploring, modeling, and visualizing data a natural one. Complete data analyses can often be represented in just a few lines of code.

In addition, thousands of others have contributed additional functionality to the R language by creating add-on "packages" for use by the 2 million users of R worldwide. One such functionality is “BIOCONDUCTOR package”.

Bioconductor (http://www.Bioconductor.org/) is based primarily on the R programming language, but also contains contributions of other programming languages. The functional scope of the Bioconductor includes the analysis of all types of genomic data. Bioconductor methods use advanced programming techniques or R resources (such as transparent data base or network access) to minimize memory requirements and integrate with diverse resources. More than 500 Bioconductor packages have been developed for expression and other microarrays, sequence analysis, imaging, and other domains.

Packages provide functionality beyond that available in base R. There are over 4000 packages in CRAN (comprehensive R archive network) and more than 600 Bioconductor packages. Packages are contributed by diverse members of the community; they vary in quality (many are excellent) and sometimes contain idiosyncratic aspects to their implementation.

The main Bioconductor packages used in high throughput sequence analysis is given in Table 3

Procedure	**Packages**
Annotation	GenomicFeatures, VariantAnnotation
Data representation	IRanges ,GenomicRanges, GenomicFeatures, Biostrings, BSgenome, girafe
Input / Output	ShortRead, Rsamtools, rtracklayer, VariantAnnotation, R453Plus1Toolbox.
Alignment	Rsubread, Biostrings, R Bowtie, RSamtools, QuasR
ChIP-seq etc.	BayesPeak, baySeq, ChIPpeakAnno, chipseq, ChIPseqR, ChIP-sim, CSAR, DiBind, MEDIPS, mosaics, NarrowPeaks, nu-cleR, PICS, PING, REDseq, Repitools, TSSi.
Database	SRAdb

Table 3 : Bioconductor packages used in High Throughput analysis.

3.4. Comparative analysis of *Mycobacterium tuberculosis* strain

The new sequencing technologies generated high throughput data in the form of short reads. Aligning these short reads was the computing bottleneck for the analysis of next generation sequencing data. Thus, several alignment softwares have developed to open this bottleneck. They all output in SAM (Sequence Alignment/Map) format, which has become standard alignment format and supported by many other alignment viewers and generic variant callers. Bowtie aligns Illumina reads to human genome at a rate of over 25 million reads per hour. It indexes the reference genome Burrows Wheeler Transform (BWT; Li and Durbin, 2009) to keep its footprint small. Thus it extends the previous Burrows-Wheeler techniques with a novel quality-aware backtracking algorithm that permits mismatches. To achieve even greater alignment speed multiple processor cores can be used simultaneously. Therefore, speed do not hurdles data processing pipeline (Li and Homer, 2010). Bowtie is free, open source software available from its website (http://Bowtie.cbcb.umd.edu/; Langmead *et al*, 2009).

Previously, all the strains of *Mycobacterium tuberculosis* were considered genetically identical but the data from recent studies showed greater genetic diversity than originally expected (Hirsh *et al*, 2004; Gagneux *et al*; 2006, Hershberg *et al*, 2008). This diversity may relate to various phenotypic consequences from virulence to emergence of drug resistance. Duplication may be present in either drug resistant or drug sensitive strain. Multiple duplication of same genic region was reported in different strains of *M.tuberculosis*. Gene duplication might play role in expression of other genes or the evolution of new gene function (Weiner *et al*, 2012). A greater knowledge of the diversity present in *M. tuberculosis* and MTBC strains can also lead to deeper understanding of the biological consequences associated with strain variability. The variation in circulating *M. tuberculosis* isolates has been critical for identification of strains, outbreaks and changes within the population. Association was also seen with phenotypic properties that are relevant in terms of the disease, such as transmission potential, immunological response and manifestation of the disease (Nicol and Wilkinson, 2008).

Genetic diversity within bacterial species is usually generated by mutations and by the exchange of genetic material. The process of HGT (Horizontal Gene Transfer) is thought to be an important driver of bacterial evolution in both pathogenic and non-pathogenic bacteria (Becq *et al*, 2007). Horizontally transferred genes can be acquired in clusters known as genomic islands or pathogenicity islands that can be identified by characteristics that distinguish them from the host genome, such as GC content, flanking nucleotide repeats and insertion elements. In the case of *M. tuberculosis*, there is evidence of ancient gene transfer events that could have taken place in a progenitor tubercle bacilli pool before the clonal expansion that gave rise to the MTBC (Gutierrez *et al*, 2005).There is a growing recognition that there is substantial genetic diversity among isolates. At the level of SNVs changes can be either synonymous (sSNV) or non-synonymous (nsSNV) and this diversity has been undeniably useful for typing and defining evolutionary relationships among strains.

Comparing DNA sequences of related organisms show an excess of transitional substitutions over the transversional substitutions. This bias could be due to relatively high rate of mutation of methylated cytosine to thymine. Excluding these mutations associated with the methylation left
The genome of *Mycobacterium tuberculosis* contains two large gene families named as PE and PPE. Though the function of this gene family is not confirmed, it is thought to be cell surface associated and involved in virulence and antigenic variation of bacteria. PE/PPE genes in H37Rv, comprises 10% of organism's genomic coding potential (Cole *et al*, 1998). It had previously been shown that non-PE/PPE families show the significant variation. However the variations in PE-PPE gene family showed further increase as compared to those non-PE/PPE genes (McEvoy, 2012).

The variation of amino acid substitution rates in proteins depends on several variables. Among these, the protein's expression level, functional category, essentiality, or metabolic costs of its amino acid residues may play an important role. In their analysis, the rate of non-synonymous substitution was shown to correlate with expression levels independently of the rate of synonymous substitution. The results also suggested an important direct influence of expression levels, or at least codon usage bias for translation optimization, on the rates of non-synonymous substitutions in bacteria. It was also indicated that when a control for this variable is included, essentiality plays no significant role in the rate of protein evolution in bacteria (Rocha and Danchin, 2004).

The availability of complete genome sequences of *M. tuberculosis* H37Rv and related strains combined with advances in high-throughput experimental technologies have led to tremendous advances in our knowledge of the evolution, population biology, and virulence of *M.tuberculosis*, which has been applied to the development of new diagnostic tests, better drugs and vaccines against TB.

METHODOLOGY

Download FASTA files of 3 Supercontigs of drug resistant *Mycobacterium tuberculosis* strain

Supercontigs of 3 *M. tuberculosis* strains: AMXW, AAKR, AQQC were downloaded from Whole Genom*e* Shotgun Sequencing Project of EMBL-EBI (http://www.ebi.ac.uk/genomes/wgs.html). AMXW, AQQC, AAKR are the clinical isolates of drug resistant Malaysian, Indian and USA strain respectively.

AMXW (accession number AMXW01000001:AMXW01000158)

AQQC (accession number AQQC01000001:AQQC01000182) and

AAKR (accession number AAKR01000001:AAKR01000160).

Genomes of all the three AMXW,AQQC, AAKR strains were analyzed for the single nucleotide variations with reference to H37Rv strain.

Obtain fASTA file of reference strain :-

Mycobacterium tuberculosis H37Rv used as reference strain. Obtained the fasta file of complete genome of *Mycobacterium tuberculosis* H37Rv (Accession number NC_000962.3) from NCBI (http://www.ncbi.nlm.nih.gov/genome/).

Bioconductor installation in R :-

R is a free software programming language and a software environment for statistical computing and graphics. The R language is widely used among statisticians and data miners for developing statistical software and data analysis. The source code for the R software environment is written primarily in C, Fortran, and R. R is freely available under the GNU General Public License, and pre-compiled binary versions are provided for various operating systems. R uses a command line interface; however, several graphical user interfaces are available for use with R.

Bioconductor provides tools for the analysis and comprehension of high-throughput genomic data. Bioconductor uses the R statistical programming language, and is open source and open development.

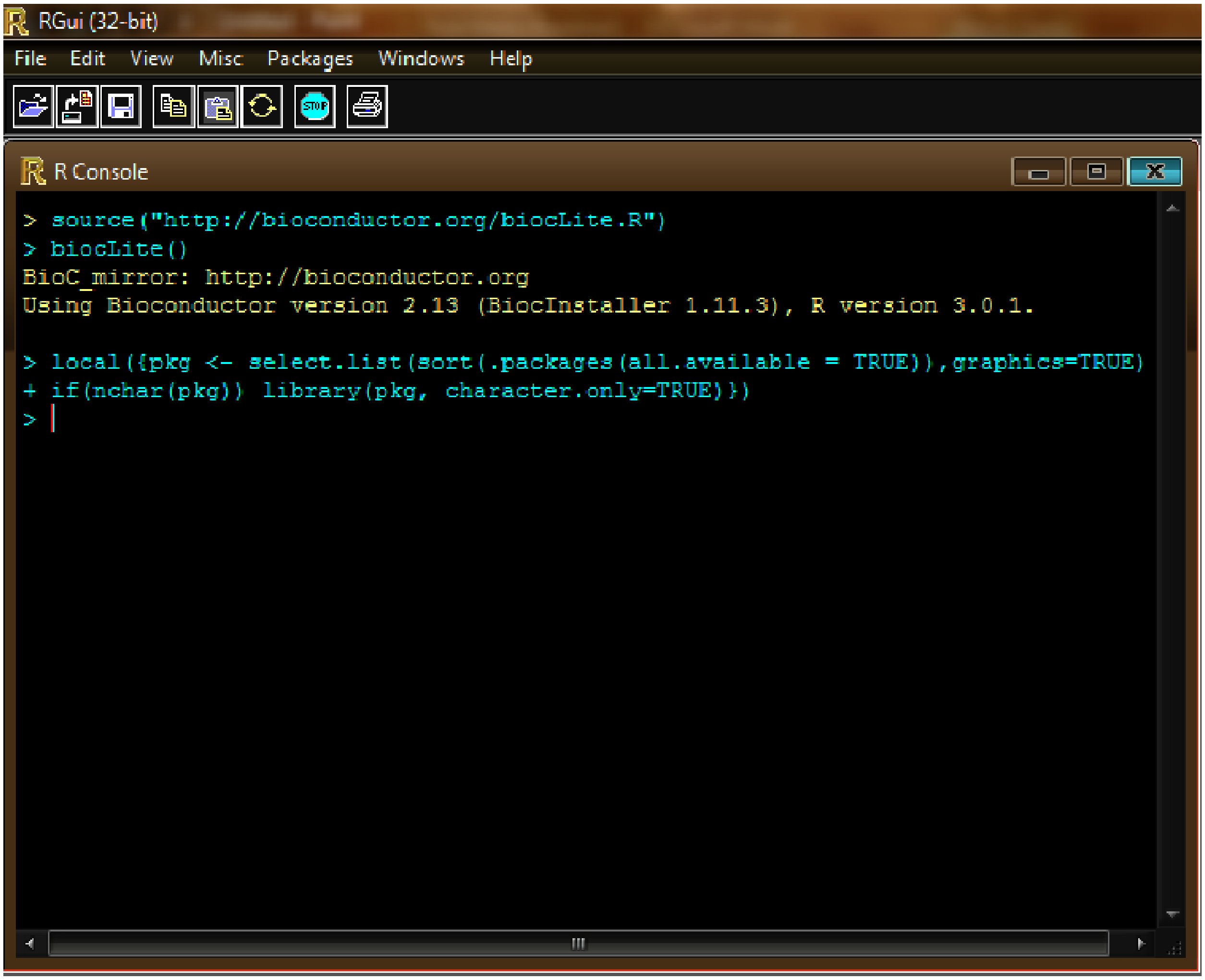
Install R, then get the version of Bioconductor by starting R and entering the commands :-

source(http://Bioconductor.org/biocLite.R)
biocLite( )

There are above 500 different software packages in Bioconductor .

Install the package according to the use using commands in R. Use the biocLite.R script to install Bioconductor packages. To install core packages, type the following in an R command window:

source(http://Bioconductor.org/biocLite.R)
biocLite("package name")



```
> source("http://bioconductor.org/biocLite.R")
> biocLite()
BioC_mirror: http://bioconductor.org
Using Bioconductor version 2.13 (BiocInstaller 1.11.3), R version 3.0.1.

> local({pkg <- select.list(sort(.packages(all.available = TRUE)),graphics=TRUE)
+ if(nchar(pkg)) library(pkg, character.only=TRUE)})
> 
```

Figure 4. Showing the R window and installation of Bioconductor in R.

Generate the short read of 40 bp long stretch for each contig :

Breaking the contig i.e AMXW, AQQC, AAKR into short read of fixed length. Lets take it as 40 bp long.

Biostrings is Bioconductor package used in the analysis. It is memory efficient string containers, string matching algorithms, and other utilities, for fast manipulation of large biological sequences or sets of sequences.

#fasta file of short reads of produced for each strain

```
>AMXW = read.DNAStringSet("AMXW.fa")
>length(AMXW)
>xaa = DNAStringSet(AMXW[[1]], start = seq(1,width(AMXW[1]) - 39,1 ), width =40)
>amxwfull40mers=xaa;
 >for (i in 2:158)
{
temp = DNAStringSet(AMXW[[i]], start = seq(1,width(AMXW[i]) - 39,1 ), width =40) ;
amxwfull40mers = append(amxwfull40mers, temp)
}
>length(amxwfull40mers)
>names(amxwfull40mers) = as.character(seq(1,4270064,1))
>write.XStringSet(amxwfull40mers,file="amxwfull40mers.fa",format ="fasta")
```

It generates the output file name amxwfull40mers.fa

Indexing the reference genome using Bowtie :-

Bowtie is an ultrafast, memory-efficient short read aligner geared toward quickly aligning large sets of short DNA sequences (reads) to large genomes. Bowtie indexes the genome with a Burrows-Wheeler index to keep its memory footprint small. Bowtie can also output alignments in the standard SAM format. Bowtie runs on the command line under Windows, Mac OS X, Linux, and Solaris.

Bowtie-build builds a Bowtie index from a set of DNA sequences. It indexes the genome to keep its memory footprint small. Bowtie build outputs a set of 6 files with suffixes .1.ebwt, .2.ebwt, .3.ebwt, .4.ebwt, .rev.1.ebwt , and .rev.2.ebwt. These files together constitute the index: they are all that is needed to align reads to that reference. The original sequence files are no longer used by Bowtie once the index is built.

Indexing the genome H37Rv in the command prompt using the command :-

```
bowtie-build H37Rv.fa H37Rv
```

Then the output generates 6 files which constitute the index :-

1. H37Rv.1.ebwt,
2. H37Rv.2.ebwt,
3. H37Rv.3.ebwt,
4. H37Rv.4.ebwt,
5. H37Rv.rev.1.ebwt,
6. H37Rv.rev.1.ebwt.

Indexing of H37Rv used for conversion of SAM to BAM :

Alignment of reference strain H37rv with query strains AMXW, AQQC, AAKR generated output in SAM format. SAM is Sequence Alignment Map which is a TAB-delimited text format contains header section (optional) and alignment section. Alignment section contains 11 mandatory fields and several other optional fields. Thus to improve performance, these SAM files were further manipulated by using SAM tools (Li *et al*, 2009) which converts the SAM format into BAM (Binary Alignment Map) format and generates alignment in per position format, so that we were able to detect the number of reads of query strain representing single base position in the reference genome sequence. SAM format contains the detailed information of alignment, while the BAM format contains the same data in compressed, indexed and binary form to achieve fast random access.

For indexing the reference H37Rv in FASTA format following command was used in command prompt :

```
samtools faidx H37Rv.fa
```

This command results into creation of 'H37Rv.fa.fai' file which was further used to convert SAM format into BAM format. BAM files were then sorted and indexed to achieve fast retrieval of alignments overlapping a specified region without going through the whole alignments. Therefore, the alignment was generated in per position format.

Alignment using Bowtie :-

Bowtie aligns the short read i.e. amxwfull40mers to the reference H37Rv.fa using the RBowtie, Rsamtools etc. package in R platform (figure 4). Install the RBowtie, Rsamtools, Genomic Ranges and IRanges in R.

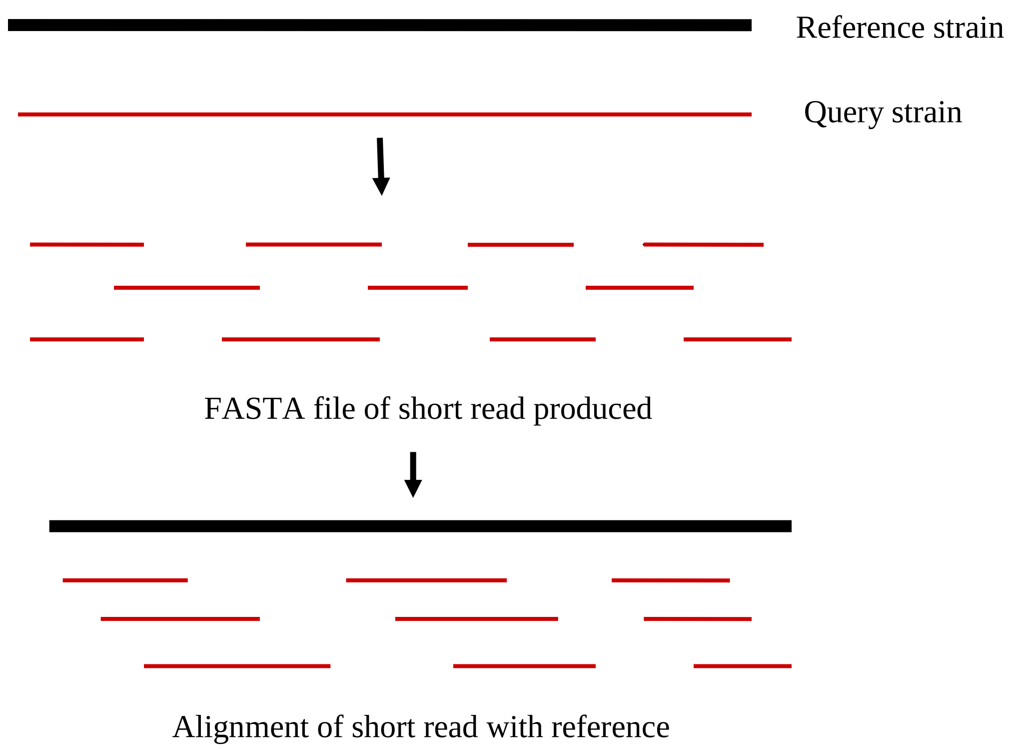


Figure 4. Representation of alignment using Bowtie.

The parameter used for bowtie alignment is as follows :-

- -p: the no. processors used for alignment
- -n: no. of allowed mismatches
- -l: length of short reads
- --sam: print alignments in SAM format

Bowtie command in R platform using Rbowtie package :-

```
>system("bowtie –n 3 –l 40 –sam H37Rv.fa amxwfull40mers.fa amxwfull40mers.sam")
```

- #-p 6: no. of processors used
- #-n 0: Bowtie run on identity
- #-l 40: length of short read
- #--sam: print in SAM format

Generate file amxwfull40mers.sam.

It generates the output in SAM format. SAM contains 11 mandatory fields. These fields always appears in the same order.

The 11 mandatory fields according to their order are as follows:-

- QNAME
- FLAG
- RNAME
- POS
- MAPQ
- CIGAR
- RNEXT
- PNEXT
- TLEN
- SEQ
- QUAL

These 11 mandatory fields either take valus as ‘0’ or ‘*’.

‘*’ means information is unavailable .

Convert SAM to BAM using SAM Tools :-

SAM Tools provide various utilities for manipulating alignments in the SAM format, including sorting, merging, indexing and generating alignments in a per-position format.

Samtools utilities

- import: SAM-to-BAM conversion
- view: BAM-to-SAM conversion and subalignment retrieval
- sort: sorting alignment
- merge: merging multiple sorted alignments
- index: indexing sorted alignment
- faidx: FASTA indexing and subsequence retrieval
- tview: text alignment viewer
- pileup: generating position-based output and consensus/indel calling.

SAM is a TAB-delimited text format whereas Bam is binary format. SAM is easy to understand, easy to parse, easy to generate and easy to check for errors. However, SAM is a bit slow to parse. SAM convert into BAM as BAM is human readable version stores the same data but in a compresses, indexed and binary form.

Scripts for converting SAM into BAM :-

```
>system(“samtools import H37Rv.fa.fai amxwfull40mers.sam amxwfull40mers.bam)
```

Output : generates file amxwfull40mers.bam

BAM is the compressed binary version of the Sequence Alignment/Map (SAM) format, a compact and index-able representation of nucleotide sequence alignments.

Sort and create index of BAM file :-

Sorting is used for ordering of the alignments. An SAM/BAM file can be sorted by the reference coordinates, by query names, or unsorted. However, most operations on the alignments only work on a BAM sorted by the leftmost reference coordinate. Such an order is crucial to data processing on a stream and to indexing. A command-line tool is provided to sort an unsorted BAM in the required order.

Scripts for sorting :-

```
>system(“samtools sort amxwfull40mers.bam amxwfull40mers_sorted”)
```

Output :- amxwfull40mers_sorted.bam

The sort command appends amxwfull40mers.bam to amxwfull40mers_sorted, creating a BAM file of alignments ordered by leftmost position on the reference assembly.

Indexing

As BAM supposed to work on spliced alignments, indexing must be efficient for alignments sppaning long distance on the reference genome.

Scripts used for indexing sorted file :-

```
>system(“samtools index amxwfull40mers_sorted.bam”)
```

Output :- file name amxwfull40mers_sorted.bam.bai

The index command generates a new file, amxwfull40mers_sorted.bam.bai, with which genomic coordinates can quickly be translated into file offsets in amxwfull40mers.sorted.bam.

Validation for Duplication :-

Length of the query strain is critical for alignment, because long read would hardly align to any region of the reference and very short read might show identity to themselves. Very short reads would represent the identical or duplicated reads, therefore, multiple reads aligned at the same region of the reference strain and script would not correctly detect the exact SNV position.

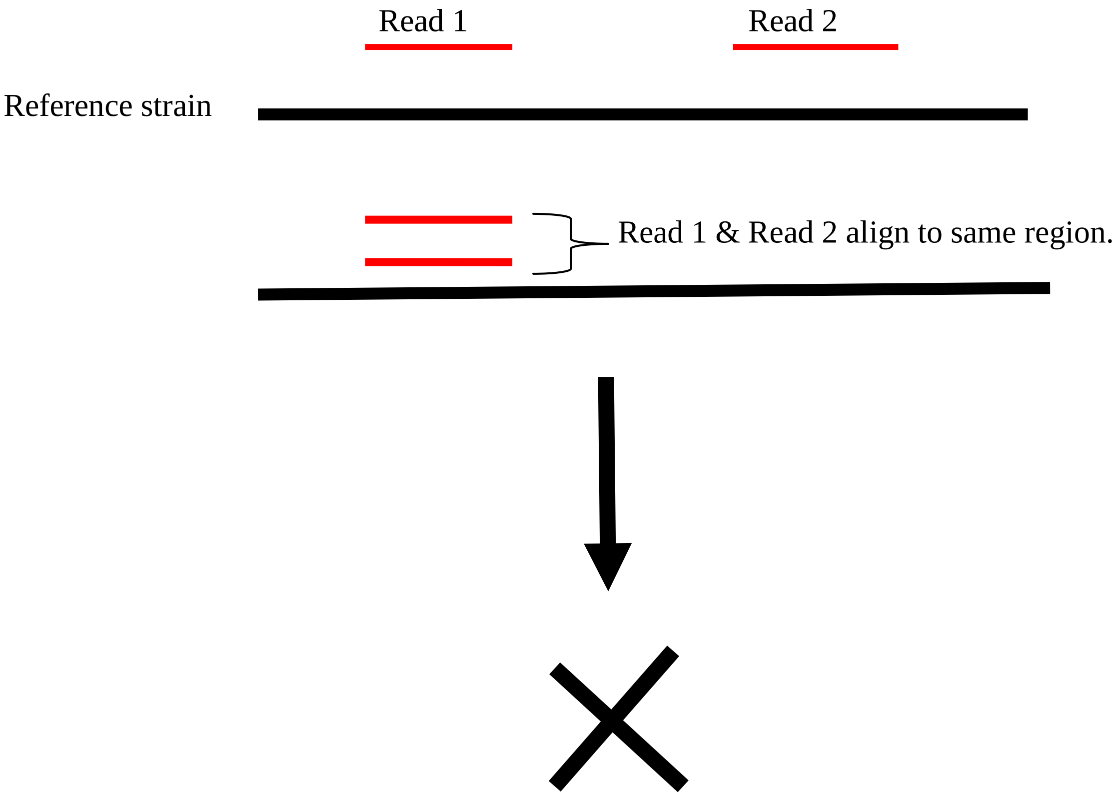


Figure 5. Duplication in the query strain.

In figure 2, read 1 and read 2 are at different paces in genome and found to be identical; therefore, align to the same region of reference genome. The position of SNVs nearby these reads could not be detected correctly. Hence, length of the read should be such that each read should align to the unique region of reference genome. This optimum read length was found to be 40.

```
#Scripts for validation of duplication:

>properalign = function(x,y)  #create properalign function.

{
```

```
x1 = read.csv(x, header=T);

xaa = GRanges("gi|57116681|ref|NC_000962.2|", IRanges(x1$end, width = 1));

                                        #myparam1 retrieve the qname and seq of GRanges object.

myparam1 = ScanBamParam(which = xaa, what = c("qname","seq")) ;

                  #same for bam file,param decides which fields and what records are imported.

tempA = scanBam(y, param = myparam1);

tempB = NULL;

for (i in 1: length(x1$end))

{

tempB = c(tempB, length(tempA[[i]]$qname))

};

return(tempB)
}
```

Example

```
>tempB1
=properalign("AHHX_identity_table.csv","ahhxfull40mers_aligned_reads_sorted.bam")

>tempC = which(tempB1 > 1)
```

If no duplicated reads were present in file, tempC would return value to zero.

SNV Detection using SAM pile up :-

SNV detection using samtool pileup command by utilising Rsamtools Bioconductor package in R platform.

Scripts used :-

```
>system(“samtools mpileup –uD –r –f H37Rv.fa amxwfull40mers_sorted.bam > bcftools view -bvcg - > var.raw.bcf "
```

Parameter :-

- ✓ –u :- tells ouput into an uncompressed file.
- ✓ -D :- keep read depth for each sample.
- ✓ -r :- region to call SNPs (for whole genome no need to use this)
- ✓ -f :- shows that next argument be the reference genome file.
- ✓ -b :- output to BCF FORMAT
- ✓ -c :- SNP calling
- ✓ -v : variant sites (exclude monomorphic ones).
- ✓ -g :- call genotypes for each sample in addition to just calling SNPs.

In the command line above, samtools collects summary information in the input BAMs, computes the likelihood of data given each possible genotype and stores the likelihoods in the BCF format .It does not call variants.

Bcftools applies the prior and does the actual calling. It can also concatenate BCF files, index BCFs for fast random access and convert BCF to VCF. In addition, bcftools can operate on some VCFs (e.g. calling SNPs from GL-tagged VCFs), but not for all VCFs; VCF to BCF conversion is not working at the moment, either.

The pile consensus format consists of :- (Table 4)

INDEX	FIELD NAME	DESCRIPTION
1	Chr	Chromosome identifier
2	Pos	1-based coordinate
3	RBase	Reference base
4	CBase	Consensus base
5	CQual	Consensus quality
6	SNP	SNP quality
7	MAPQ	Maximum mapping quality
8	DEPTH	Number of reads covering this site

9	BASE	Read bases
10	QUAL	Base quality

Table 4. Pile up consensus format.

Convert BCF into VCF :-

BCF, or the binary variant call format, is the binary version of VCF. It keeps the same information in VCF, while much more efficient to process especially for many samples. The relationship between BCF and VCF is similar to that between BAM and SAM.

Script used

```
>system("samtools bcftools view var.raw.bcf | vcfutils.pl varFilter –D100 >var.flt.vcf")
```

This line converts the BCF file into a VCF file (a flat text file rather than a binary, making it a lot easier to view), and then we pipe that into vcfutils.pl with the varFilter -D100 option, which filters out SNPs that had read depth higher than 100 (we don't want to trust SNPs at sites with super high coverage, because they might be represent variation between variable copy number repeats, i.e., the reads that map to this location in the reference are actually from duplicated sites in your sample; you can–and should–change this parameter based on the kind of coverage you have in your dataset, e.g., -D500).

The Variant Call Format (VCF) is the emerging standard for storing variant data(figure 6). Originally designed for SNPs and short INDELs, it also works for structural variations.

VCF consists of a header section and a data section. The header must contain a line starting with one '#', showing the name of each field, and then the sample names starting at the 10th column. The data section is TAB delimited with each line consisting of at least 8 mandatory fields (the first 8 fields in the table below). The FORMAT field and sample information are allowed to be absent.

The file lists the chromosome, position, ID, reference allele at that nucleotide position, and alternate alleles detected in our dataset (across all samples). It also tells you the "Quality" – which is basically a measure of how confident Samtools is that there really is a SNP there (higher is better) – and whether or not that SNP passed the quality filters. The "Info" field tells you various statistics about each position; the information in this field can vary, but what exactly each symbol (e.g., NS, DP, etc.) should be explained in the file header. The "Format" field tells you what type of data are found in the rest of the fields (there should be one additional field for each sample that you ran through Samtools). For example, GT:GQ:DP means that the sample fields for this position tell you the genotype, genotype quality, and depth (coverage) of each sample that you fed into Samtools; again, explanations of these symbol names (GT, GT, DP) can be found in the header of your VCF file.

Col	**Field**	**Description**
1	CHROM	Chromosome name
2	POS	1-based position. For an indel, this is the position preceding the indel.
3	ID	Variant identifier. Usually the dbSNP rsID.
4	REF	Reference sequence at POS involved in the variant. For a SNP, it is a single base.
5	ALT	Comma delimited list of alternative seuqence(s).
6	QUAL	Phred-scaled probability of all samples being homozygous reference.
7	FILTER	Semicolon delimited list of filters that the variant fails to pass.
8	INFO	Semicolon delimited list of variant information.
9	FORMAT	Colon delimited list of the format of individual genotypes in the following fields.
10	Sample(s)	Individual genotype information defined by FORMAT.

Table 5. VCF format

Generate table for SNVs detected :-

Alignment of query with reference strain result in mismatches. These SNV detected through BOWTIE and SAMtools command line interface . now these SNVs are generated in table format to analyse the number of SNV occurring .

Scripts used

```
system("samtools pileup -vcf H37Rv.fa amxwfull40mers_sorted.bam > raw.pileup")
```

❖ H37Rv.fa.	Fasta formatted file of the reference genome.
❖ amxwfull40mers_sorted .bam	Sorted BAM formatted file, from the alignments.
❖ raw.pileup	Output pileup formatted, with consensus calls.
❖ -c	Calls the consensus base at each position.
❖ -v	Show positions that do not agree with ref.fa.
❖ -f	Reference sequence, ref.fa (in Fasta format)

```
snps <- readPileup("raw.pileup", variant="SNP")
                                                  #read SNPs
```

```
indels <- readPileup("raw.pileup", variant="indel")
                                                                              #read indels

write.table(as.data.frame(snps), "snps.csv")
                                                                        # generate SNP  excel sheet
```

These scripts would generate a table showing the SNV position and the nucleotide substitution at that position.

Example :- filter and see some SNPs
Let us see first 10 SNPs

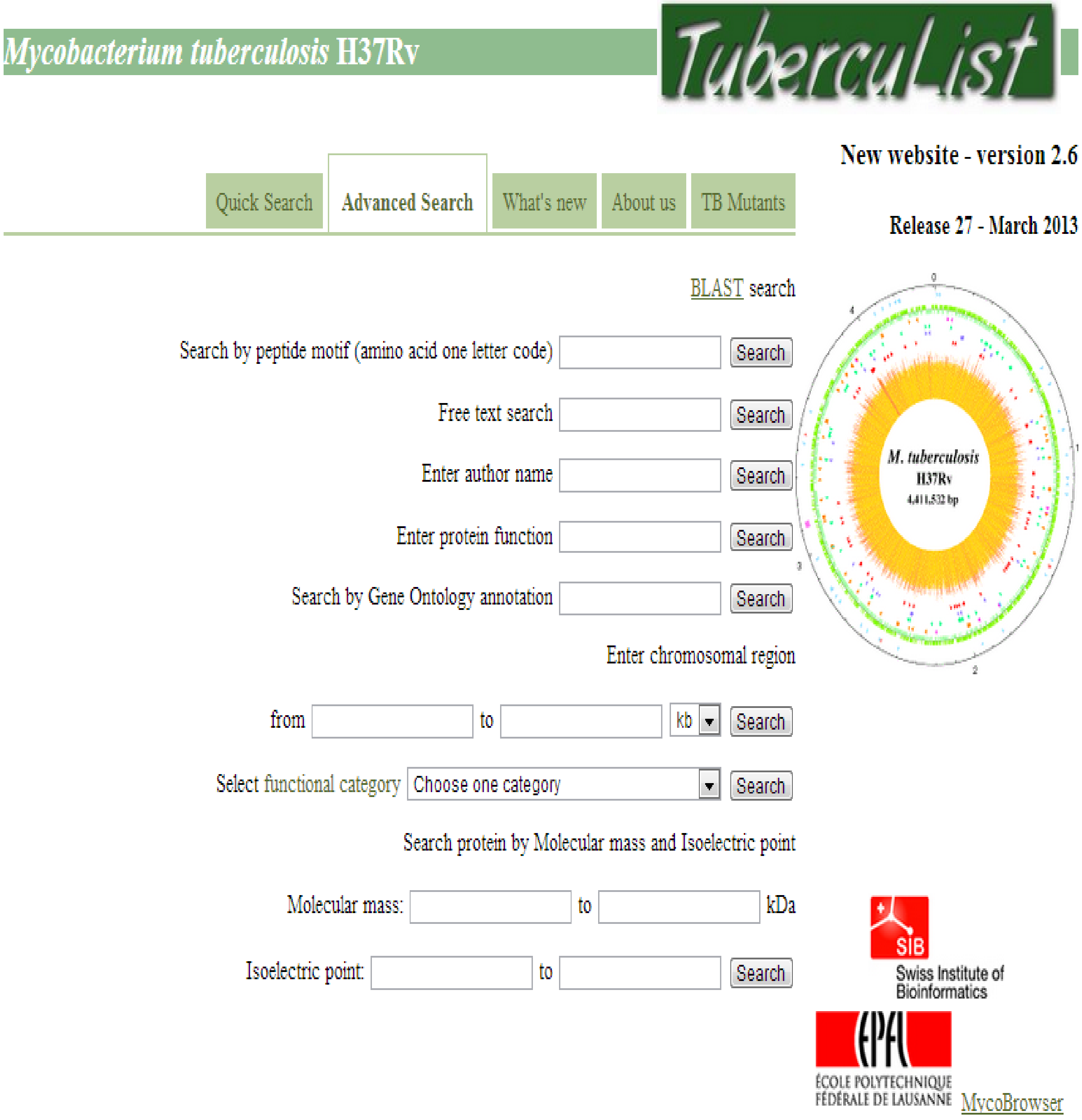
```
snps[1:10]
table(seqnames(snps))
                                                                        # table snps per chromosomesnps
<- snps[elementMetadata(snps)$coverage > 3]
                                                                       # keep only snps with coverage > 3
```

Genome analysis :-

These SNVs were further categorized into transitions and transversions on the basis of nucleotide change for each strain. The identified SNVs were further classified into genic and intergenic SNVs on the basis of their occurence in the genome. Genic SNVs were further classified into synonymous and non-synonymous SNVs. The annotation for the reference genome obtained from TubercuList and EnsemblBacteria. TubercuList page (figure) has annotated the function of each transcript into 10 categories designated as: cell wall and cell processes, conserved hypotheticals, information pathways, insertion seqs and phages, intermediary metabolism and respiration, lipid metabolism, PE/PPE, regulatory proteins, virulence, detoxification, adaptation and rest for which function was not confirmed are in collectively placed in the a category named as unknown. The list of functional category can be downloaded by submitting the start and end coordinate of the sequence. Besides, the functional category the TubercuList also contains the complete genomic information available till date about *M. tuberculosis* H37Rv strain.

Calculate dN/dS ratio :-

In genetics, the dN/dS ratio (or ω,ka/ks),is the ratio of the number of Non synonymous substitutions per non-synonymous site (K_a) to the number of synonymous substitutions per synonymous site (K_s), which can be used as an indicator of selective pressure acting on a protein-coding gene. Comparisons of homologous genes with a high dN/dS ratio are usually said to be evolving under positive selection. The dN/dS ratio is used to infer the direction and magnitude of natural selection acting on protein coding genes. A ratio greater than one implies positive or Darwinian selection; less than one implies purifying (stabilizing) selection; and a ratio of one indicates neutral (i.e. no) selection. However, a combination of positive and purifying selection at different points within the gene or at different times along its evolution may cancel each other out, giving an average value that may be lower, equal or higher than one.



Mycobacterium tuberculosis H37Rv

New website - version 2.6

Release 27 - March 2013

BLAST search

Search by peptide motif (amino acid one letter code)

Free text search

Enter author name

Enter protein function

Search by Gene Ontology annotation

Enter chromosomal region

from to

Select functional category

Search protein by Molecular mass and Isoelectric point

Molecular mass: to kDa

Isoelectric point: to

Figure 6. Showing the page of TubercuList(http://tuberculist.epfl.ch/) having the information about Mycobacterium tuberculosis strain H37Rv used as reference in the present analysis .

RESULTS

FASTA file of *Mycobacterium tuberculosis* H37Rv strain downloaded from NCBI contains 4,411,532 bp.

FASTA file of Drug resistant *Mycobacterium tuberculosis* AAKR, AMXW, AQQC strain downloaded from WGS (whole Genome Shotgun) project , they do not include any gaps. The information about the downloaded FASTA file is given in Table 6.

Super Contig Name	Strain Name	Country Name	Accession Number	No. of Contigs	Total Length (bp)
AAKR	*Mycobacterium tuberculosis* C	U.S.A	AAKR01000001:AAKR01000164	164	4,276,200
AMXW	*Mycobacterium tuberculosis* X122	Malaysia	AMXW01000001:AMXW01000196	196	4,270,064
AQQC	*Mycobacterium tuberculosis* EAI/OSDD271	India	AQQC01000001:AQQC01000182	182	4,169,405

Table 6 :- Showing the information about downloaded fasta files of the 3 Supercontigs.

All the contigs were changed into short sequence reads of 40 bp length. So, the number of short sequence of 40 bp length generated in each contig were:-

1. AAKR :- 106905
2. AMXW :- 106752
3. AQQC :- 104236

The short sequence reads of drug resistant strains of *Mycobacterium tuberculosis* taken from different countries were compared with M. tuberculosis H37Rv strain in a pair-wise manner to identify SNVs. SNVs were identified using programs BOWTIE and SAMTOOLS.

BOWTIE align these short reads of query strain AAKR, AMXW, AQQC with the reference strain *Mycobacterium tuberculosis* H37Rv and generated file in SAM format.

Bowtie output :-

1. AAKR

- # reads processed: 4269960
- # reads with at least one reported alignment: 4197043 (98.29%)
- # reads that failed to align: 72917 (1.71%)

2. AMXW :-
    - # reads processed: 4263902
    - # reads with at least one reported alignment: 4241291 (99.47%)
    - # reads that failed to align: 22611 (0.53%)

3. AQQC :-
    - # reads processed: 4162307
    - # reads with at least one reported alignment: 4101745 (98.54%)
    - # reads that failed to align: 60562 (1.46%)

Genome of these three drug resistant strains analyzed using R scripts and various Bioconductor packages and genome wide SNV table generated for each of the three strains. Here, (Table 7) shows regions of query strains that were found to be show variation with the reference H37Rv strain. It shows only 8 SNVs when AQQC Indian strain align with reference strain H37Rv.

Seqnames	Start	End	Reference Base	Consensus Base	Consensus Quality	SNP Quality	Max Mapping Quality	Cover--age
gi|448814763|ref|NC_000962.3|	1977	1977	A	G	105	190	60	40
gi|448814763|ref|NC_000962.3|	2369	2369	G	A	50	50	60	40
gi|448814763|ref|NC_000962.3|	2532	2532	T	C	69	69	60	40
gi|448814763|ref|NC_000962.3|	4013	4013	T	C	70	70	60	40
gi|448814763|ref|NC_000962.3|	6112	6112	G	T	69	69	60	40
gi|448814763|ref|NC_000962.3|	6124	6124	C	T	69	69	60	40
gi|448814763|ref|NC_000962.3|	7362	7362	G	C	70	70	60	40
gi|448814763|ref|NC_000962.3|	7585	7585	G	C	50	50	60	40

Table 7: first 8 SNPs showing when query(AQQC) align with reference strain

Rest of the alignment between AQQC strain *and Mycobacterium tuberculosis* H37Rv showing in supplementary table of Appendix.

Result of alignment between query strain (AMXW and AAKR) and Mycobacterium tuberculosis H37Rv given in the Appendix.

Total SNVs reported :-

1. AAKR :- 1623
2. AMXW :- 1327
3. AQQC :- 2139

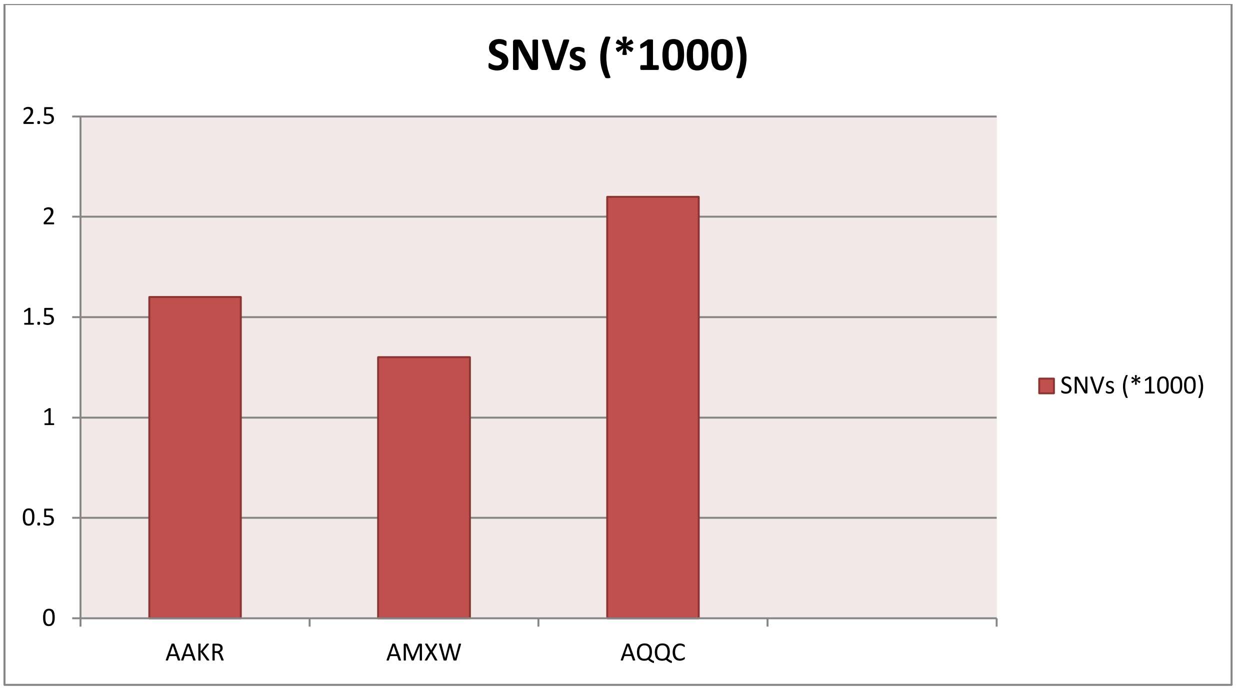


Figure 7. Showing total SNVs reported in each strain.

There is more number of SNVs in AQQC Strain compare to AAKR and AMXW suggest that AQQC are more drug resistant than the other two strain. This can be validated after analysing the synonymous mutation in AQQC.

ANALYSIS OF SNV :-

More number of SNVs are reported in genic region than intergenic region when compare the SNVs with the H37Rv tuberculosis strain database. Total number of transistions in AAKR, AMXW and AQQC were 868, 789, 1363 respectively. On the other hand the transversions in

AAKR, AMXW and AQQC were 678, 501 and 746 respectively.

1. **AAKR** :-

<u>Transistion</u> :-

- A → G -- 157
- G →A -- 290
- C →T -- 275
- T →C -- 146

Total :- 868

<u>Transversions</u> :-

- A →C -- 53
- A →T -- 27
- G →C --146
- G →T --99
- C →A --130
- C →G --129
- T →A -- 25
- T →G -- 61

Total :- 1546

2. **AMXW** :-

<u>Transistion</u> :-

- A → G -- 206
- G →A -- 192
- C →T -- 190
- T →C -- 201

Total :-789

<u>Transversions</u> :-

- A →C -- 74
- A →T -- 11
- G →C --112
- G →T -- 68
- C →A -- 72
- C →G -- 97
- T →A -- 10
- T →G -- 57

Total : - 501

3. **AQQC** :-

Transistion :-

- A → G -- 348
- G →A -- 374
- C →T -- 359
- T →C -- 282

Total :- 1363

Transversions :-

- A →C -- 81
- A →T -- 27
- G →C --147
- G →T -- 129
- C →A --127
- C →G --135
- T →A -- 21
- T →G -- 79

Total :- 746

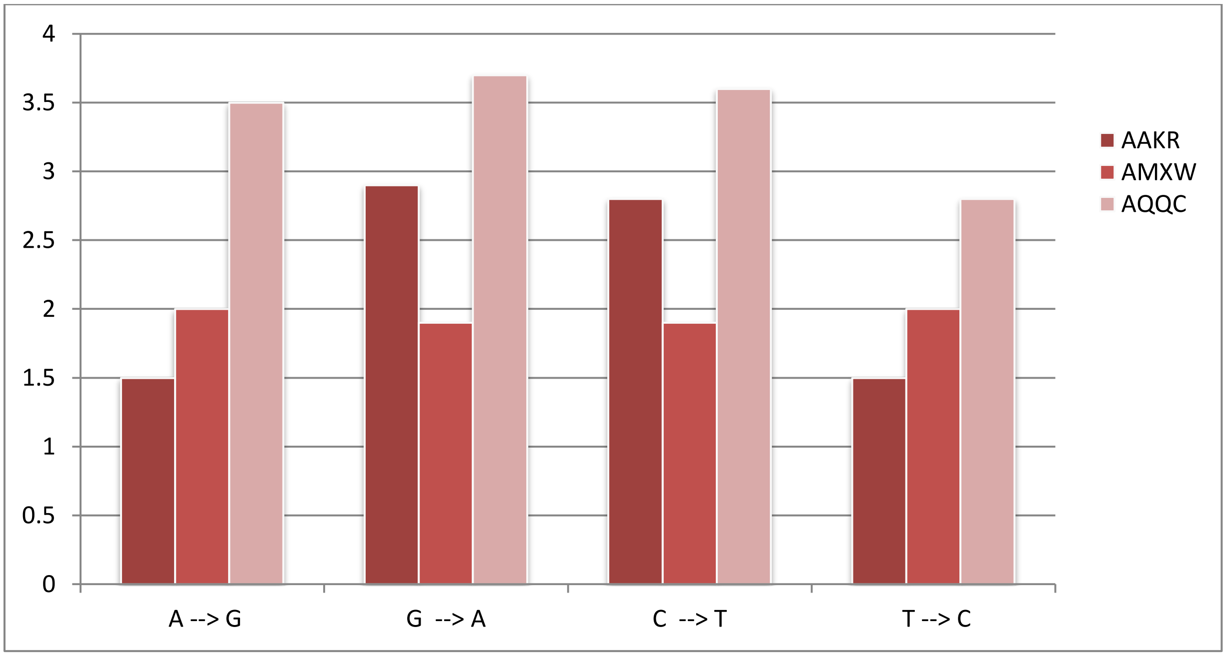


Figure 8. Showing total Transistions.

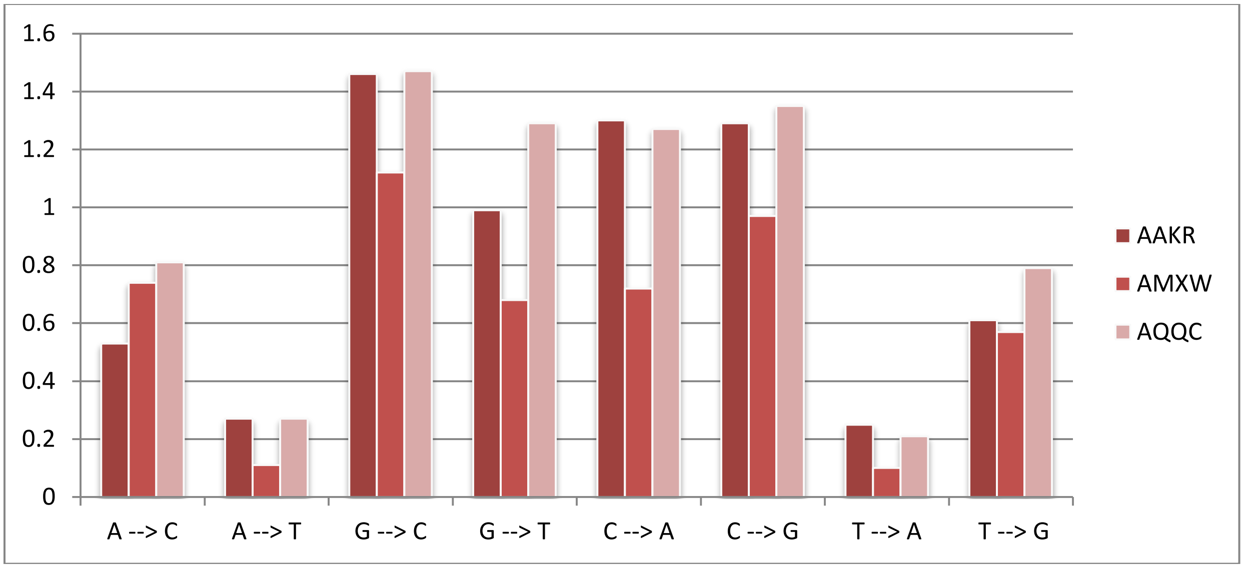


Figure 9. Showing total Transversion

From the above figure it can be concluded that there were few A→T and T→A conversion.

Ts/Tv (Transistion/Transversion)

Transistion to transversion ratio calculated in each strain.

1. AAKR :- 1.280
2. AMXW :- 1.575
3. AQQC :- 1.827

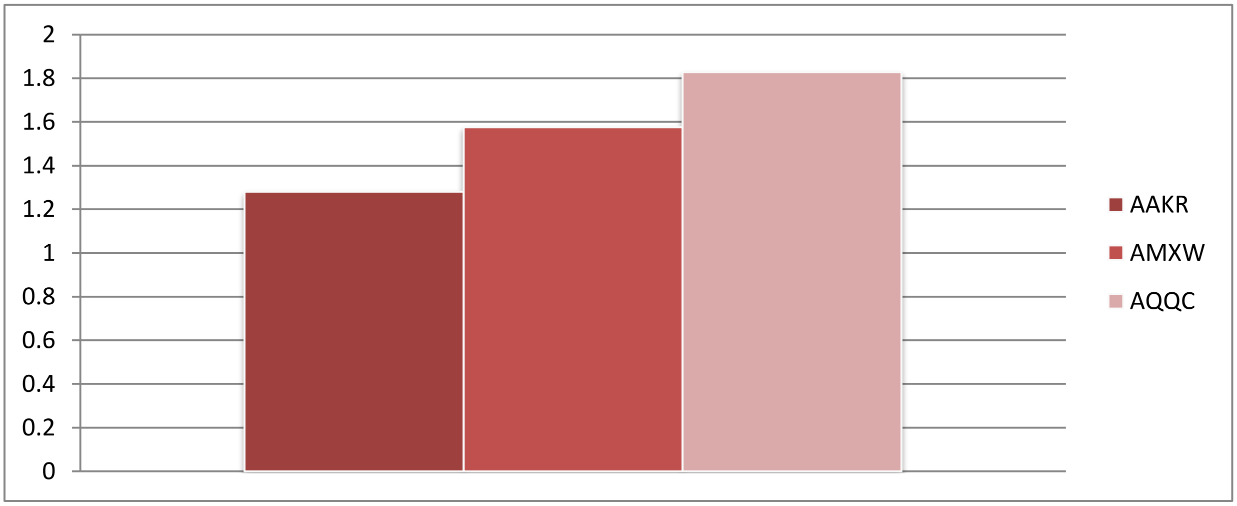


Figure 10. Showing Ts/Tv ratio in each of three strains.

This shows that there is more transistional bias in all the three strains.

Then the further analysis of the SNVs occurring in genic and intergenic region.(figure). The total number of SNVs in genic region of AAKR, AMXW, AQQC were 1207, 982 and 1659 respectively. The total number of SNVs found in intergenic region of AAKR, AMXW, and AQQC were 416, 345 and 480 resp. There is significant number of SNVs in genic region to cause different phenotypic effect , moreover it should result in variation in amino acid sequence of protein. Further categorisation of transistion and transversion in genic and intergenic region reveals that there were more number of transistion occur in both genic and intergenic region in all three strains.

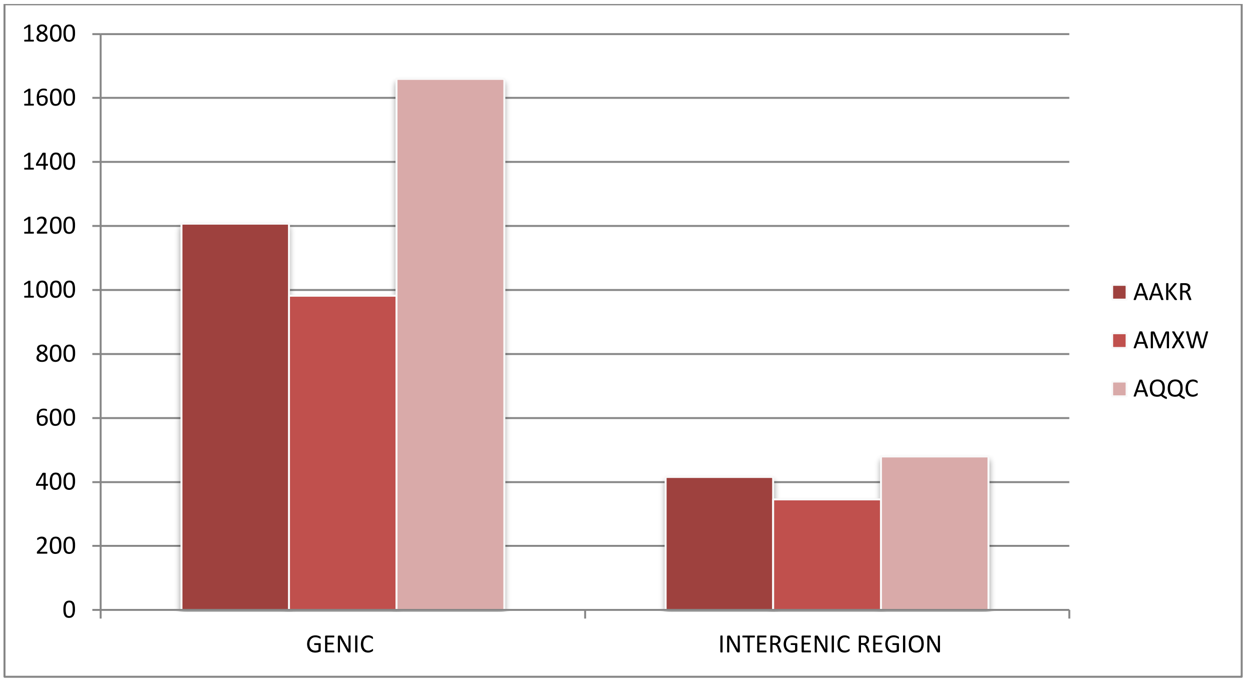


Figure 11. Showing genic and intergenic SNVs in each of the strain.

76 % of the total SNVs reported was found to be in genic region. In the genic region, Ts/Tv ratio is approx. 1.29 further shows the transistional bias in this region.

The genic SNVs were further categorized into synonymous and non-synonymous SNVs for each functional category of TubercuList and this count was normalized by the total no. of genes in each functional category. SNVs reported for unknown category of TubercuList were equal for each strain (Figure 12). Thus percent of SNVs in each category for all the three strains is 13-14% in cell wall and cell processes, 8-9 % in conserved hypotheticals, 10-11% in information pathways,9% insertion seqs and phages, 6-7% in intermediary metabolism and respiration, 17% in lipid metabolism, 13-17% in PE/PPE, 8-10% in regulatory proteins,6-7% in virulence, detoxification, adaptation, 13% in unknown and 9 % of the total SNVs were not categorized.

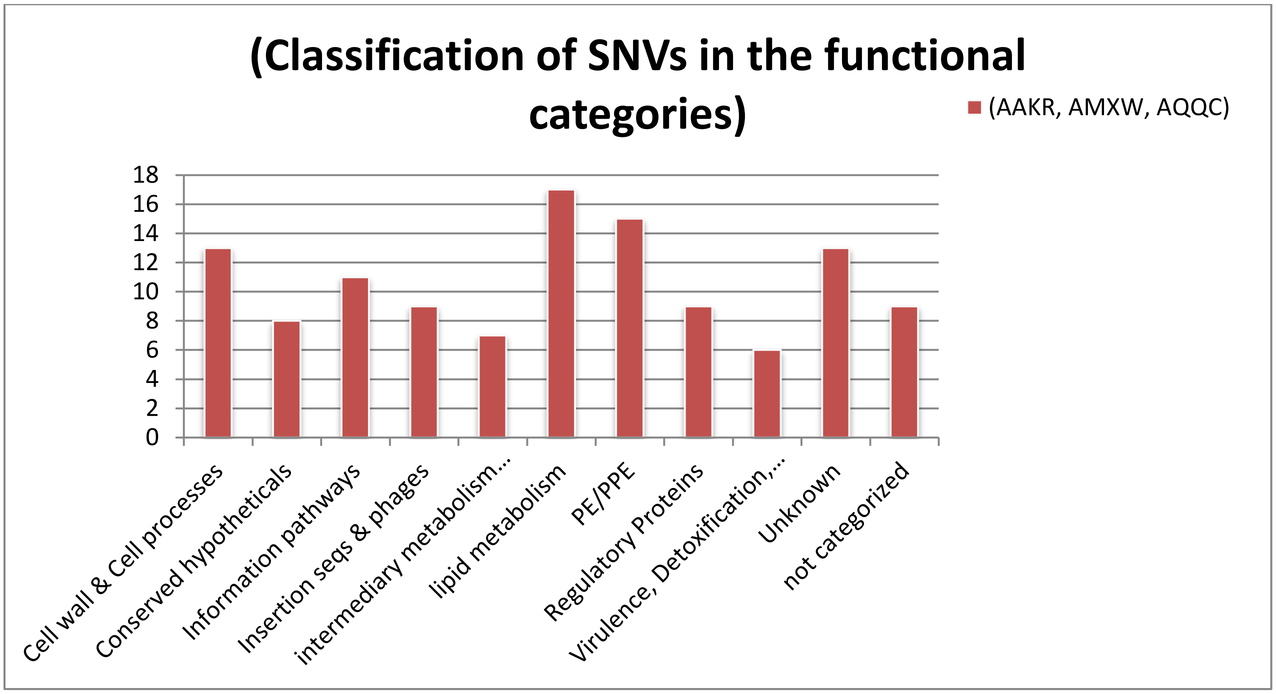


Figure 12. Showing classification of SNVs in each of the functional category.

Natural selection during evolution :-

All the SNVs on checking with synonymous and non synonymous substitution found that in all the three strains non-synonymous substitutions are greater than synonymous substitution.

In all the three strain, dN/dS ratio (ratio of the number of Nonsynonymous substitutions per non-synonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks)), is greater than 1. dN/dS ratio calculated for each functional category of the reference to show whether those non synonymous substitution can be positively selected. In all functional category, this value is greater than 1 except in “uncategorised” and “unknown” where its value is less than 1.

So, it can be concluded that changes were accepted during the process of natural selection as the dN/dS ratio in all the three strain are greater than 1.

DISCUSSION

Many *Mycobacterium tuberculosis* strain shows drug resistance. Some of the genetic variation however useful to identify mechanism involved in resistance. In the present genome wide analysis has shown that variation in *M. tuberculosis* strains may not be due to loss or gain of genes (Ochman *et al*. 2001). Rather, the changes are many, comprising mostly SNVs, small insertions/deletions, alteration in the number of tandem repeats and insertion/deletion. The variation found in the three strains i.e. AAKR, AQQC, AMXW strains of *M. tuberculosis* were more in genic region due to presence of ~91% of coding region in H37Rv strain. In almost all the strains dN/dS ratio greater than one shows that changes are allowed during natural selection. This results in the phenotypic differences in all the strains. The phenotypic properties that are relevant in terms of the disease, such as transmission potential, immunological response and manifestation of the disease (Nicol and Wilkinson, 2008).

In almost all the strains the transitional bias was maintained resulting into more number of transitions than transversions. Adenine to Thymine s and vice versa conversions are very low. Contribution of some specific nucleotide substitution in this bias was also found. Ts/Tv ratio greater than one further validate the transistional bias in all three strains.

Mycobacterium tuberculosis possesses two highly polymorphic sets of genes called the PE and PPE families. These unique families of proteins account for about 10% of the Mycobacterial genome, and is shown by the previous studies on the M.tuberculosis strain.(McEvoy,2012). The higher number of SNVs approx 15% found in PE/PPE gene family. PE/PPE accounts for multiple levels of infectious process including host immune response (Sampson,2011).

The SNVs in the PE/PPE and also in other gene region may effects its function if they fall in the active sites of that protein by changing the binding affinity of ligand of that protein.This information helpful to better understand the immunological importance of PE/PPE proteins and their roles in Mycobacterial virulence.

Comparing all the classes of genes associated with altered metabolism in organisms of different geographical origin. The SNVs belonging to functional categories are likely to affect drug sensitivity directly or indirectly. For example, entry of drugs is an important first step in their action. Changes in the membrane by lipid alteration can alter drug transport processes. Inhibition in the uptake of the drug has been postulated to be a mechanism of drug resistance in Mycobacteria (Raynaud *et al*. 1999).

High throughput approaches are very useful to analysis different strains of same species which can be further used to identify genomic variations and can further linked with phenotypes to obtain biological insights.

CONCLUSION AND FUTURE PRESPECTIVE

The emergence of drug resistance *M.tuberculosis* strain hampered the progress in the treatment of tuberculosis. Variability in the genome of *M. Tuberculosis i*s significant barrier in the treatment of tuberculosis. The fully sequenced genomic sequences can be analysed to identify

all the variations and a detailed study can be conducted to correlate genetic and phenotypic differences. This variability plays role in the virulence and survival of this pathogen but extent of the impact of this strain variability is still unclear. High protein coding efficiency of genome of *Mycobacterium tuberculosis* contributes to greater variations in the genic region. Specific nucleotide substitutions lead to the transitional bias of the strains..

In case of non-synonymous substitutions, there is likely to be an alteration in the overall structure. PE/PPE multi-gene family shows maximum variation that may contribute to the host immune response and virulence of the strains. These SNVs may lead to change in function of the resulting protein..

The larger number of SNVs observed in XDR strains suggest that this can be a potential mechanism for the evolution of drug resistance in Mycobacteria. Future work on systems-level analysis of these strains may throw a more definitive light on this important area.

In further study, all the non synonymous mutation and interactions of resulting protein and host immune response can be analyse using computational methods. After analysing effect of SNVs that cause drug resistance, we can develop new therapeutics to control the drug resistant Mycobacterium tuberculosis which helps to control the mortality due to the tuberculosis.

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- http://www.bioconductor.org/
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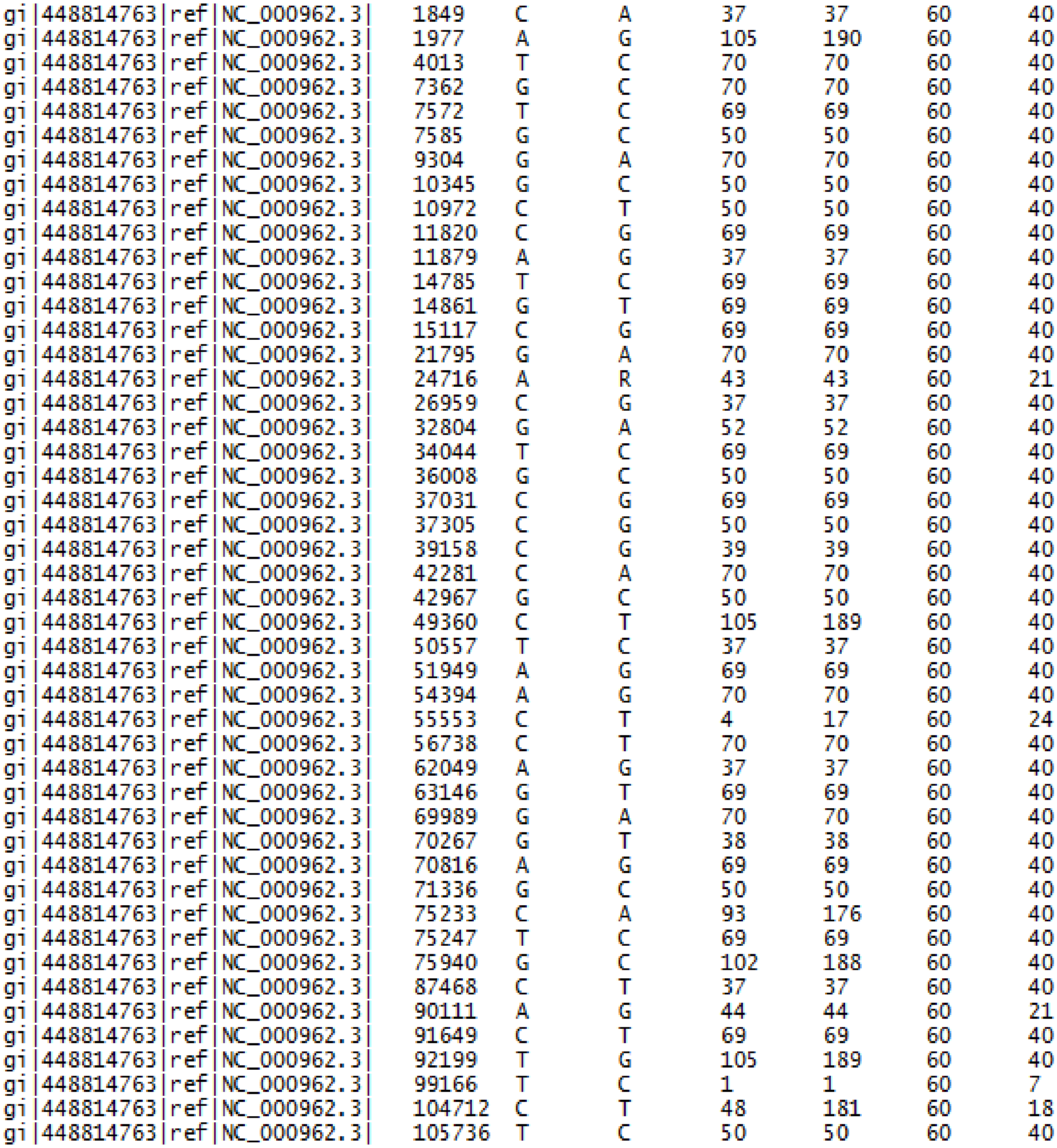
APPENDIX I :-

<u>Result of alignment of AAKR strain with *Mycobacterium tuberculosis* H37Rv</u>



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gi|448814763|ref|NC_000962.3|	21795	G	A	70	70	60	40
gi|448814763|ref|NC_000962.3|	24716	A	R	43	43	60	21
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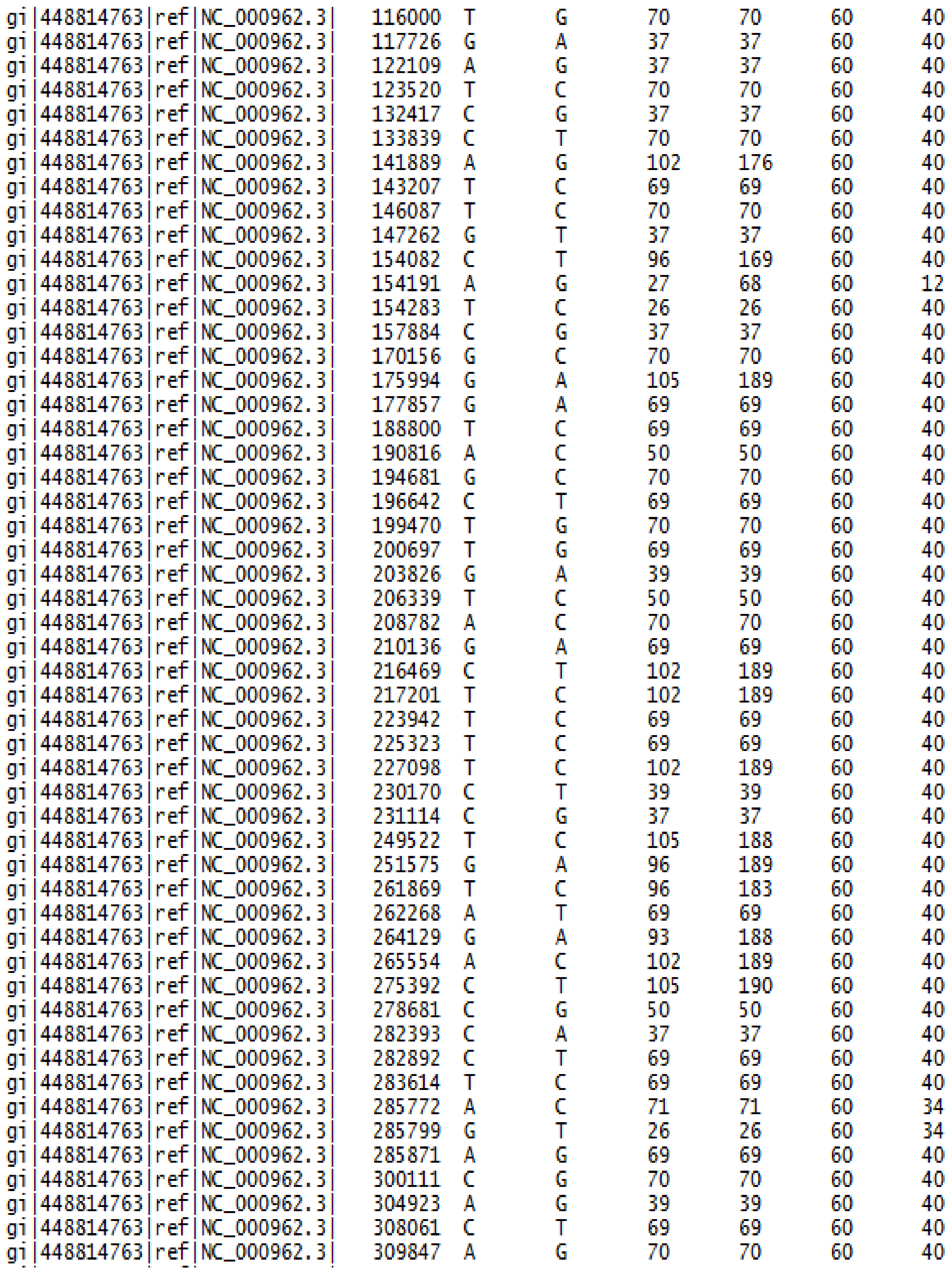

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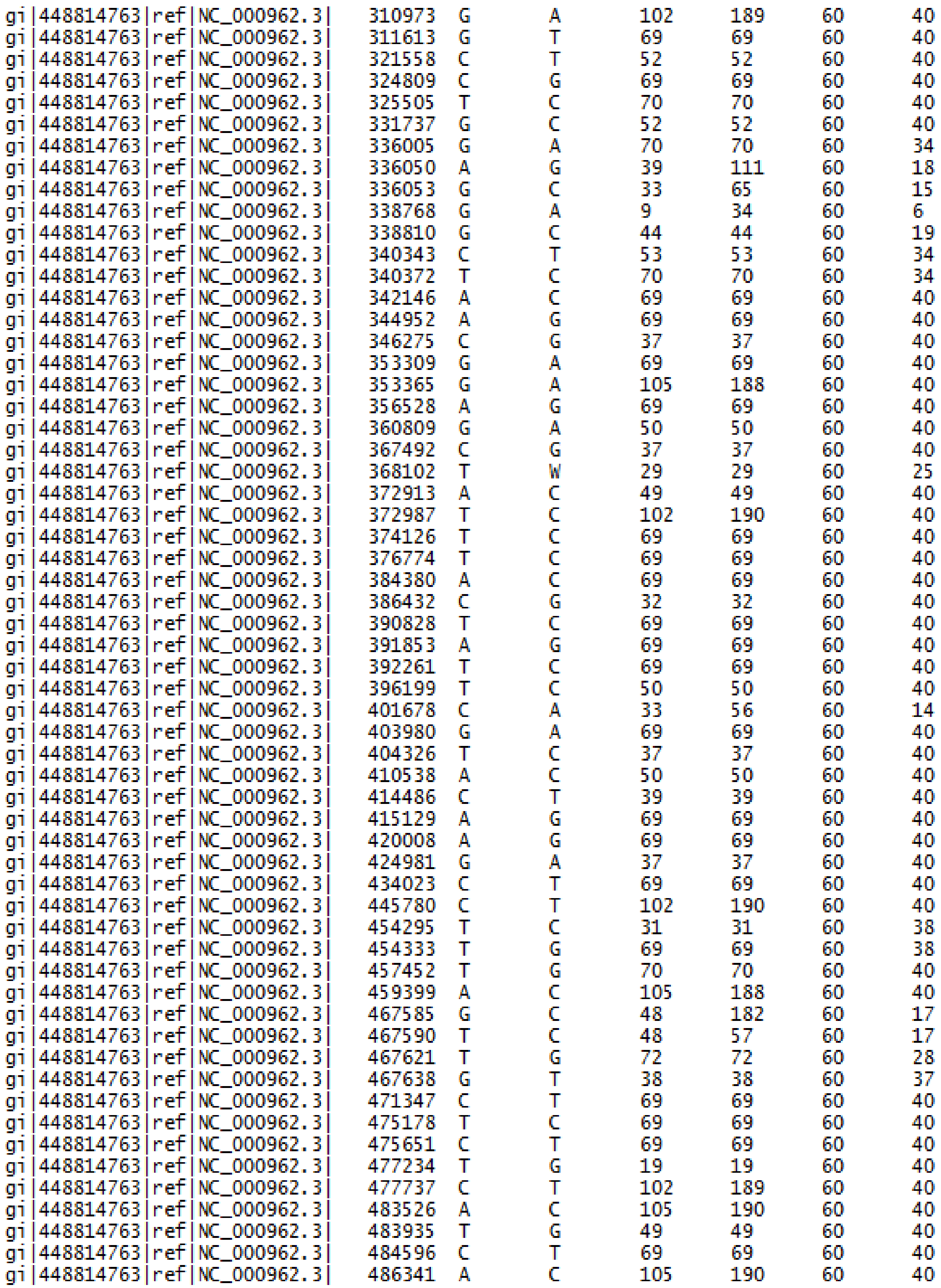

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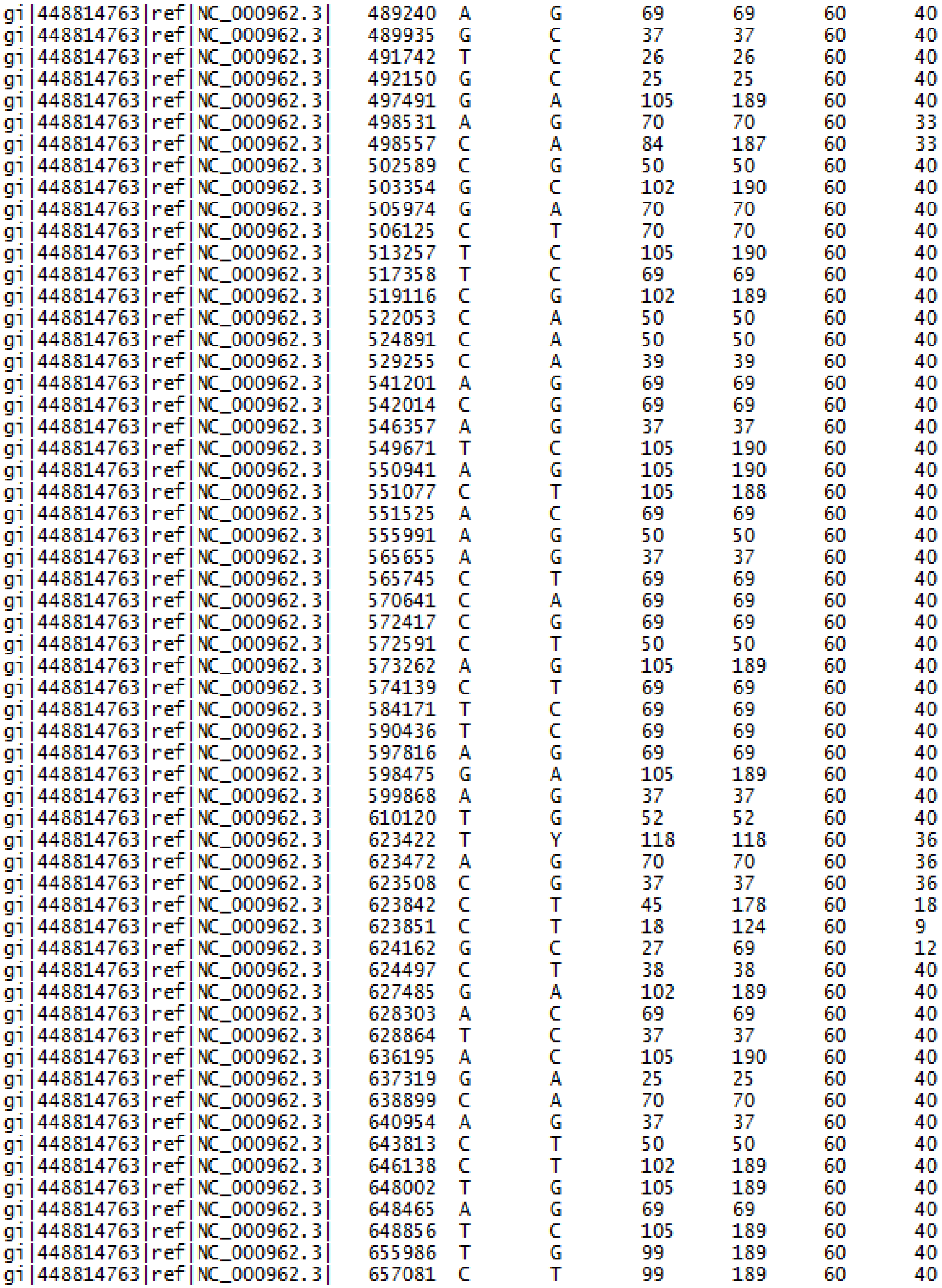

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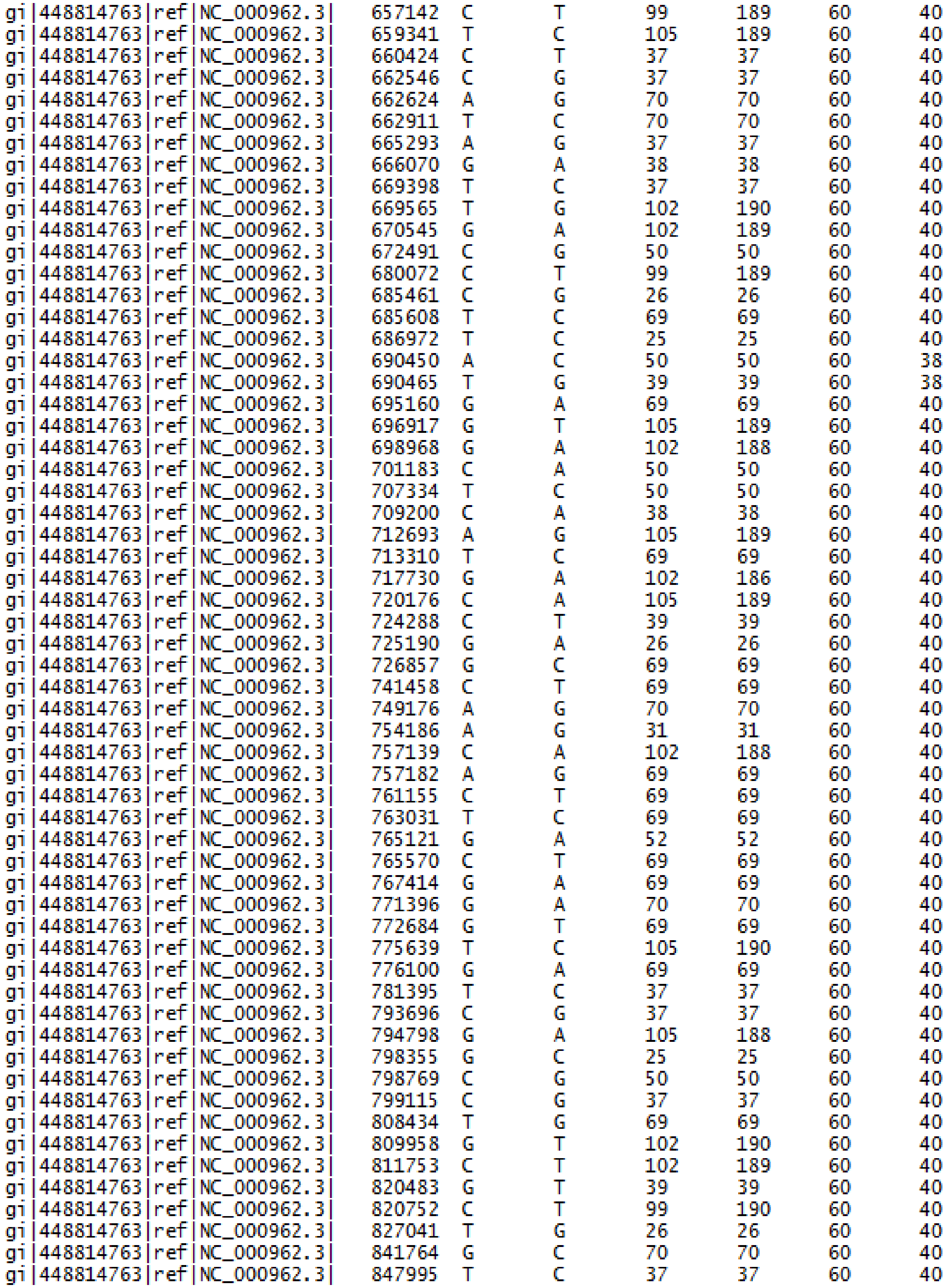

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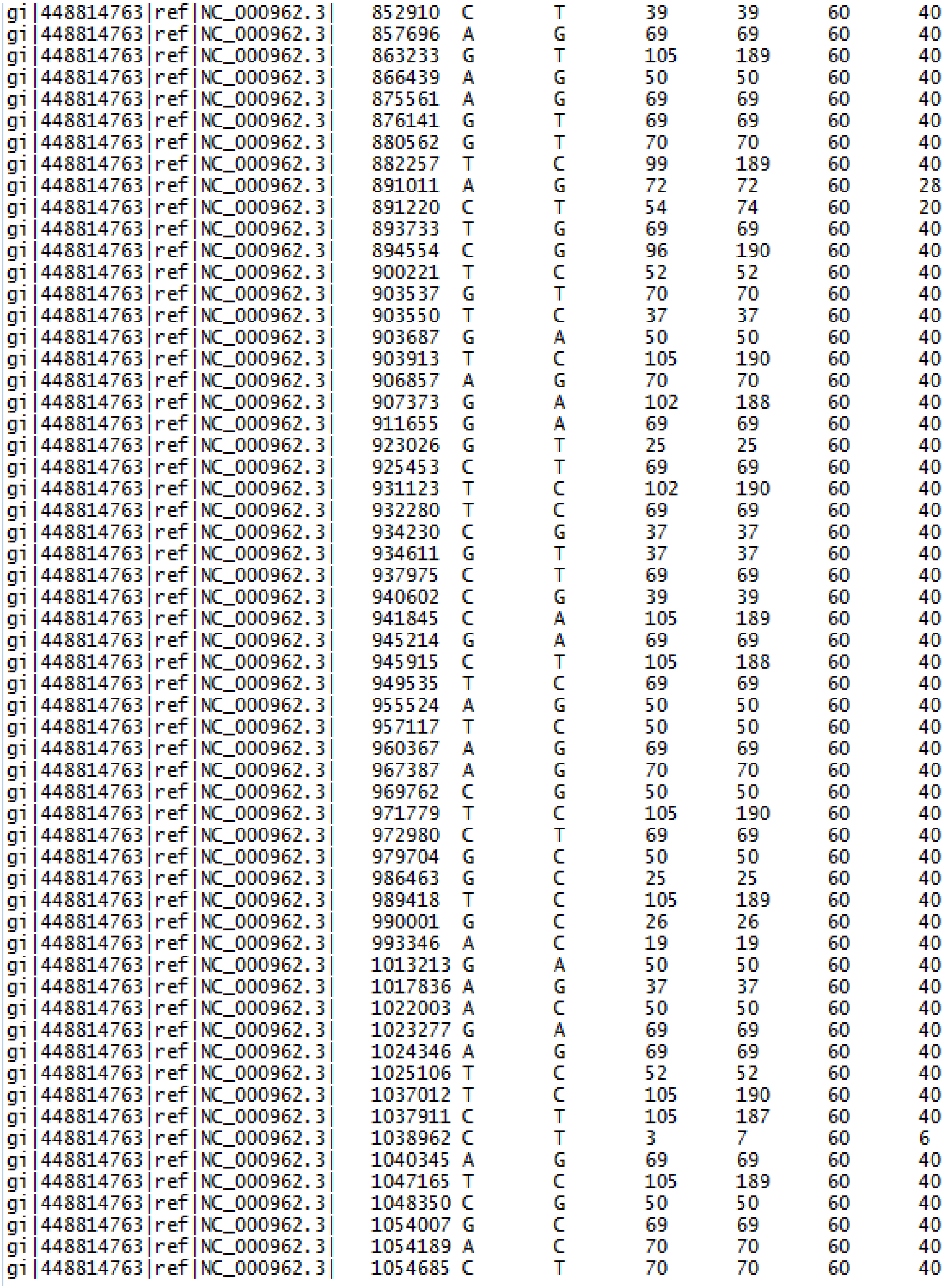

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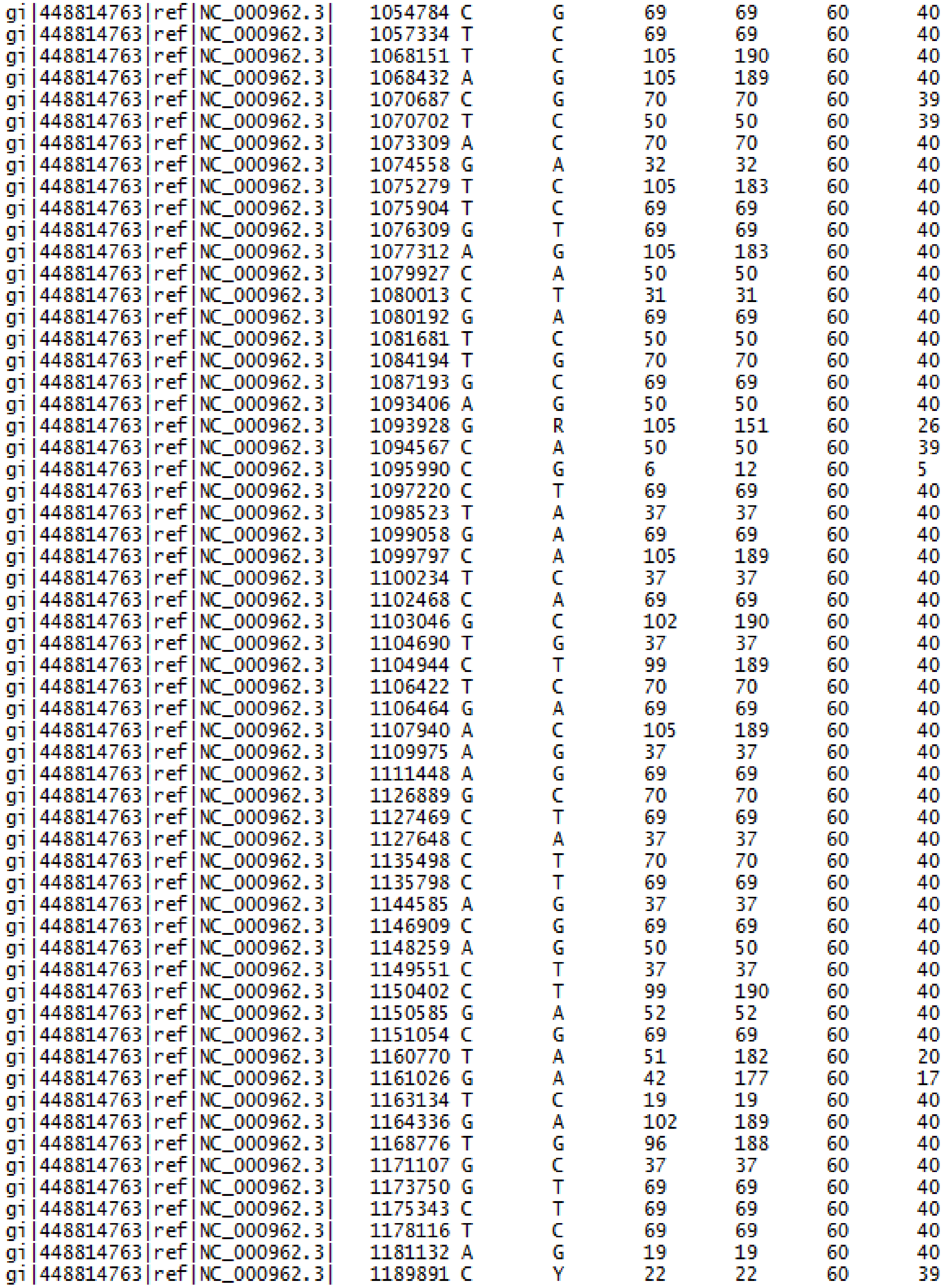

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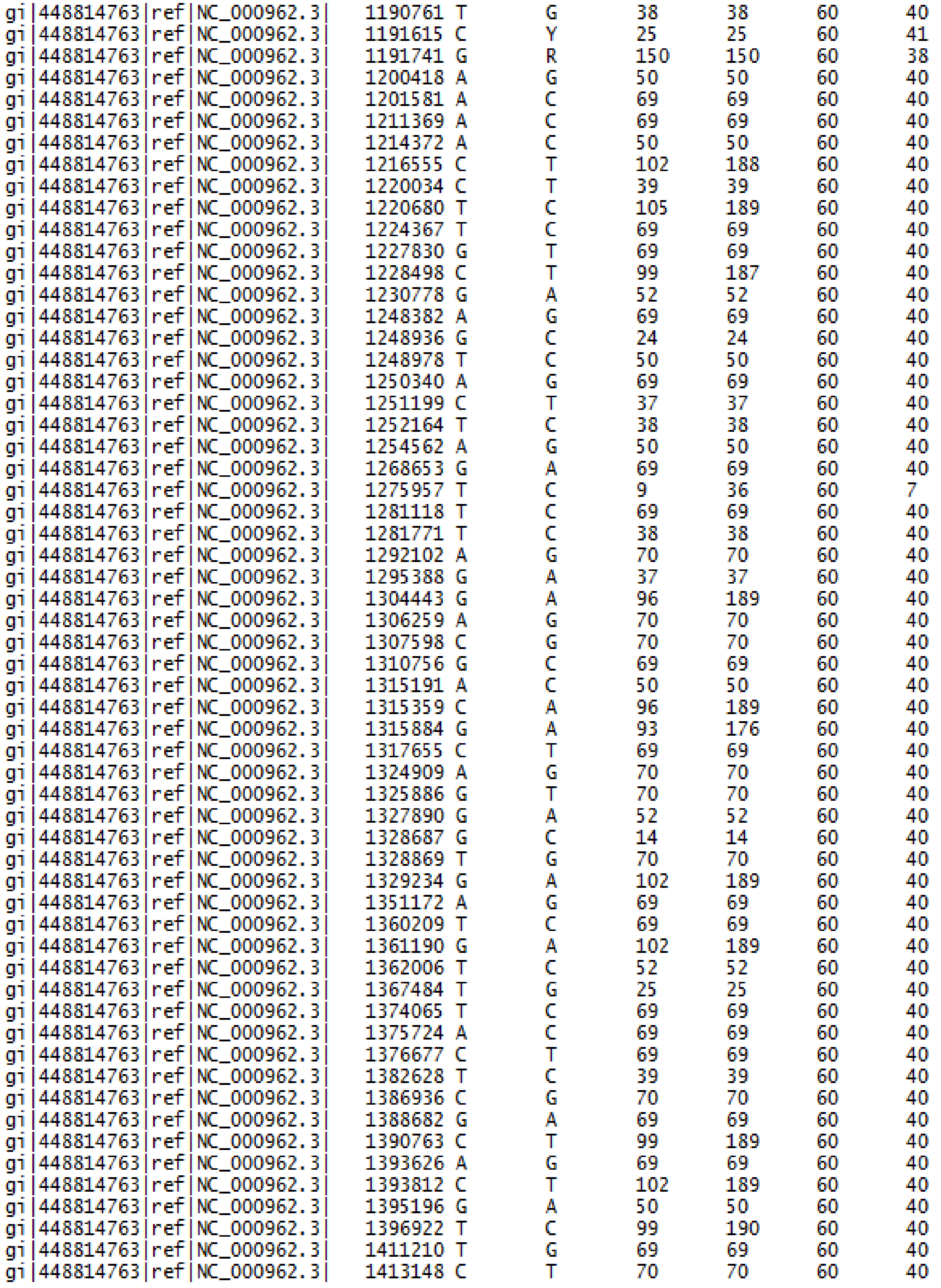

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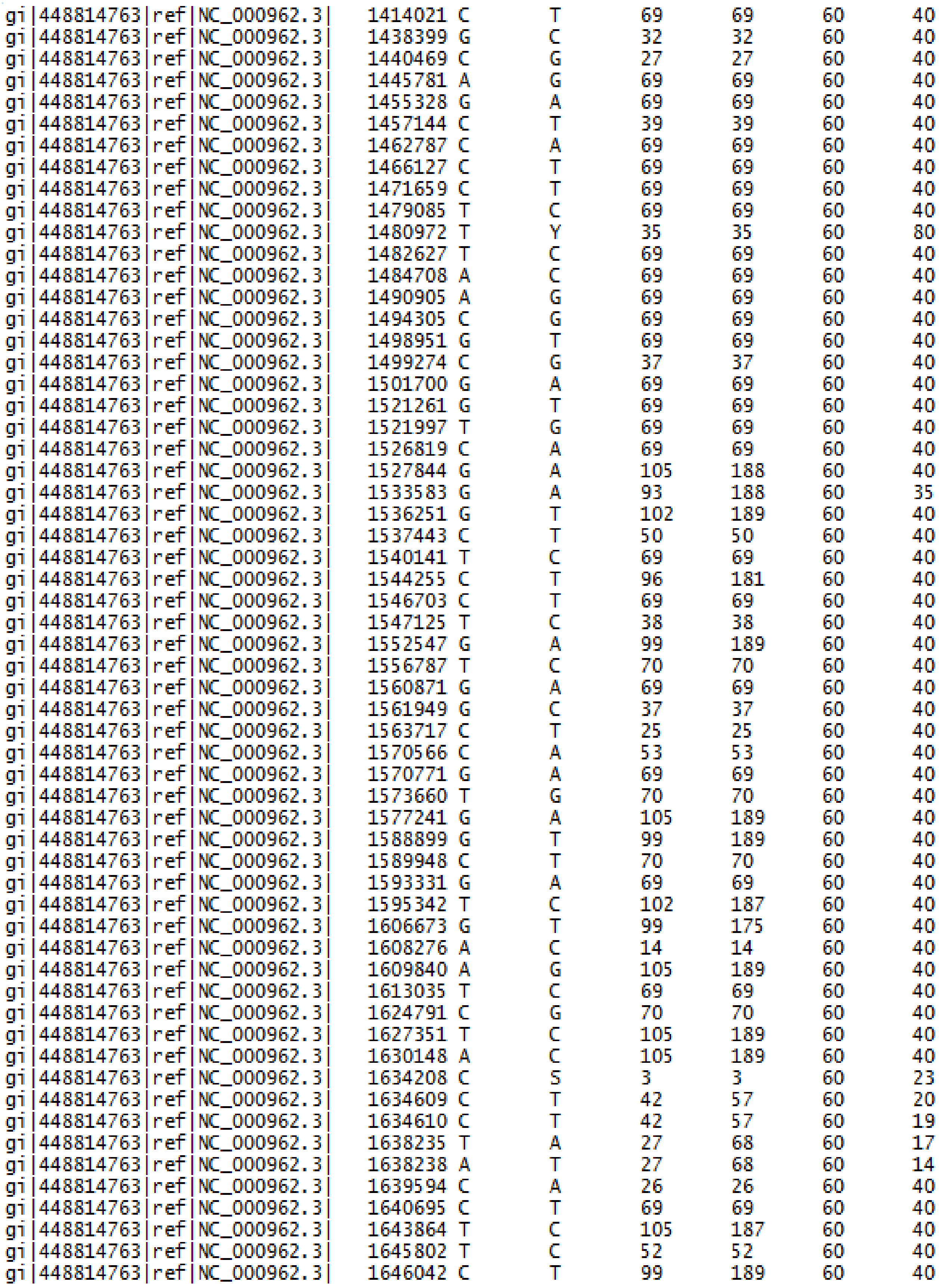

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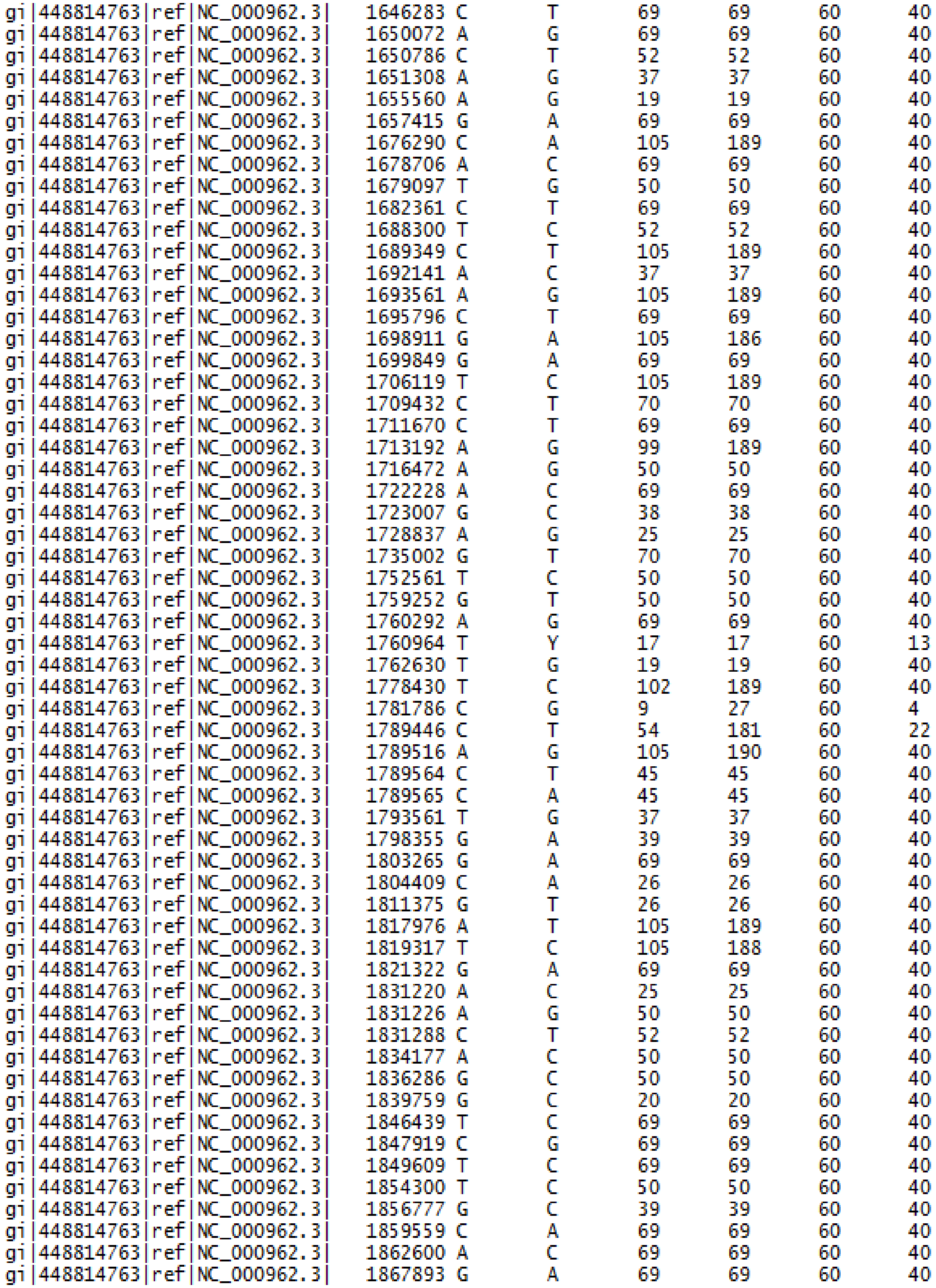

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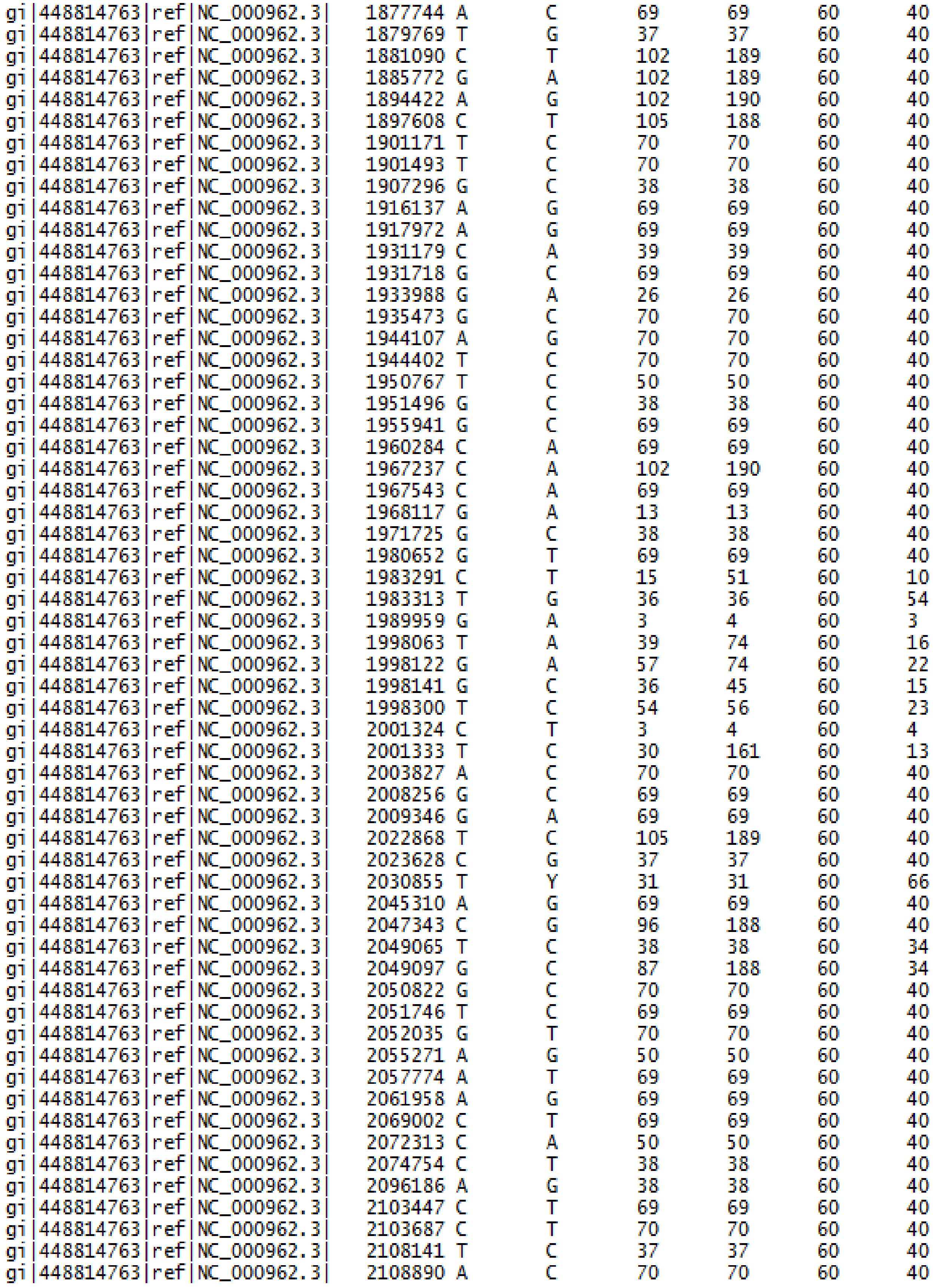

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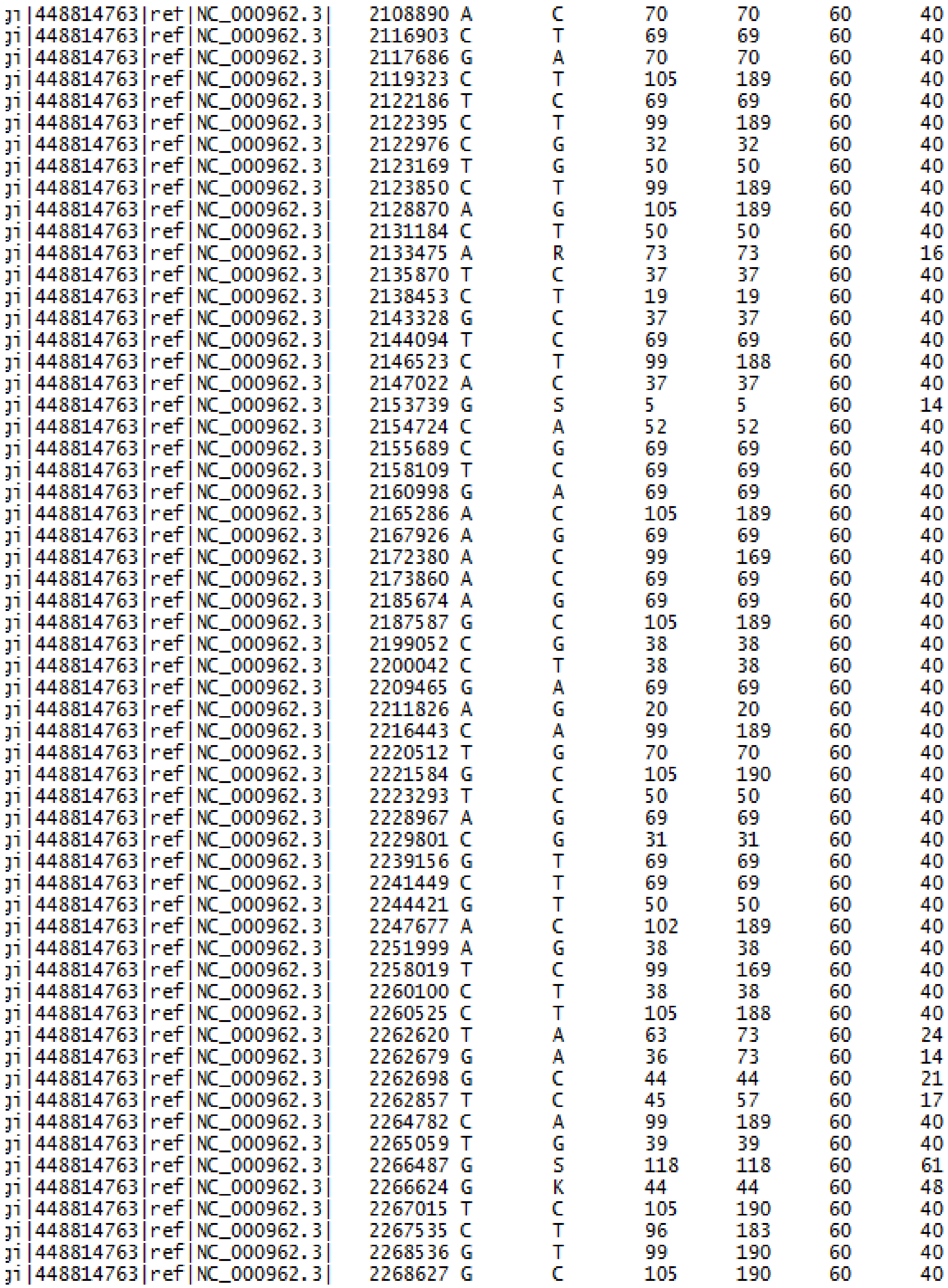

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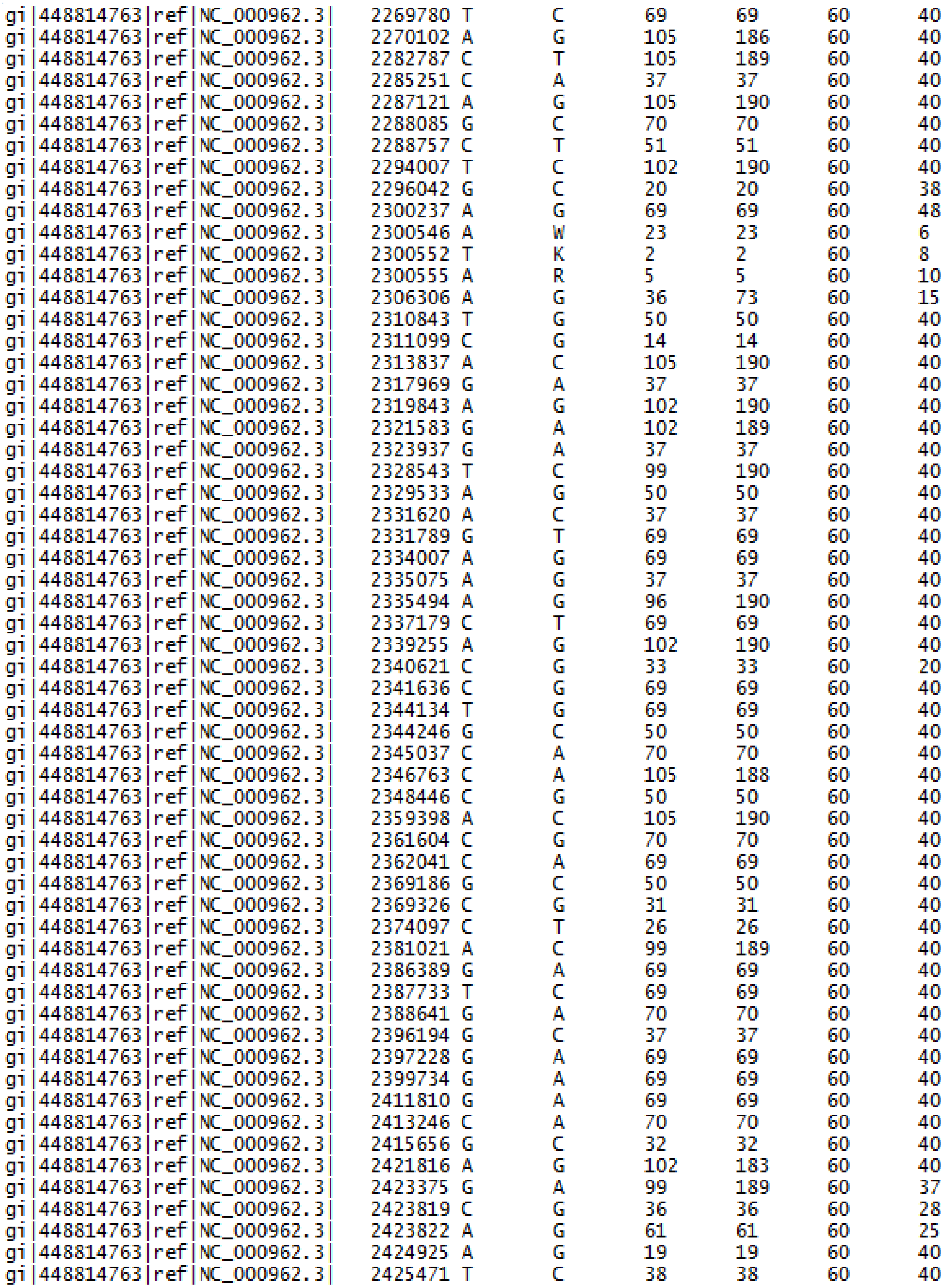

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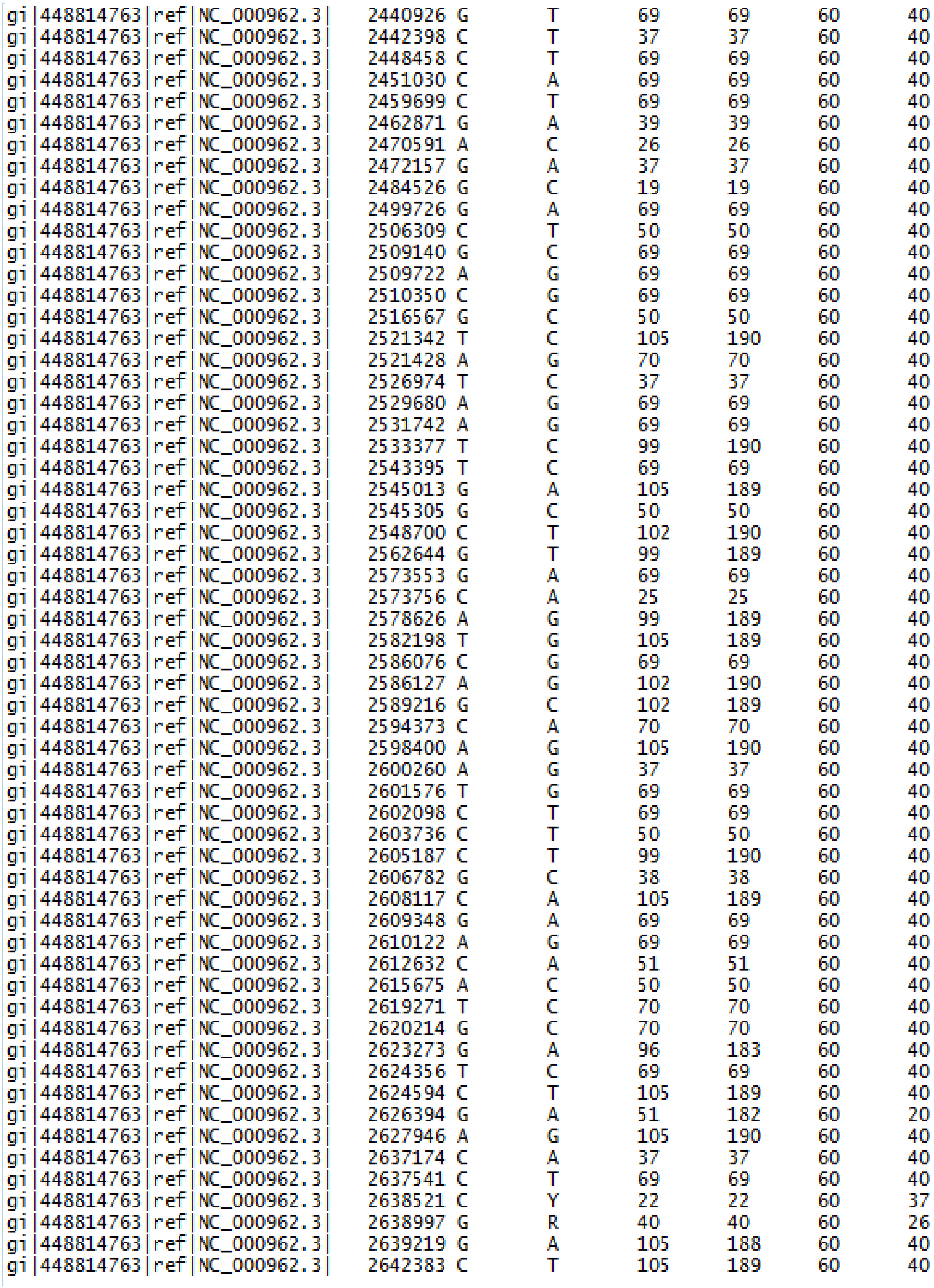

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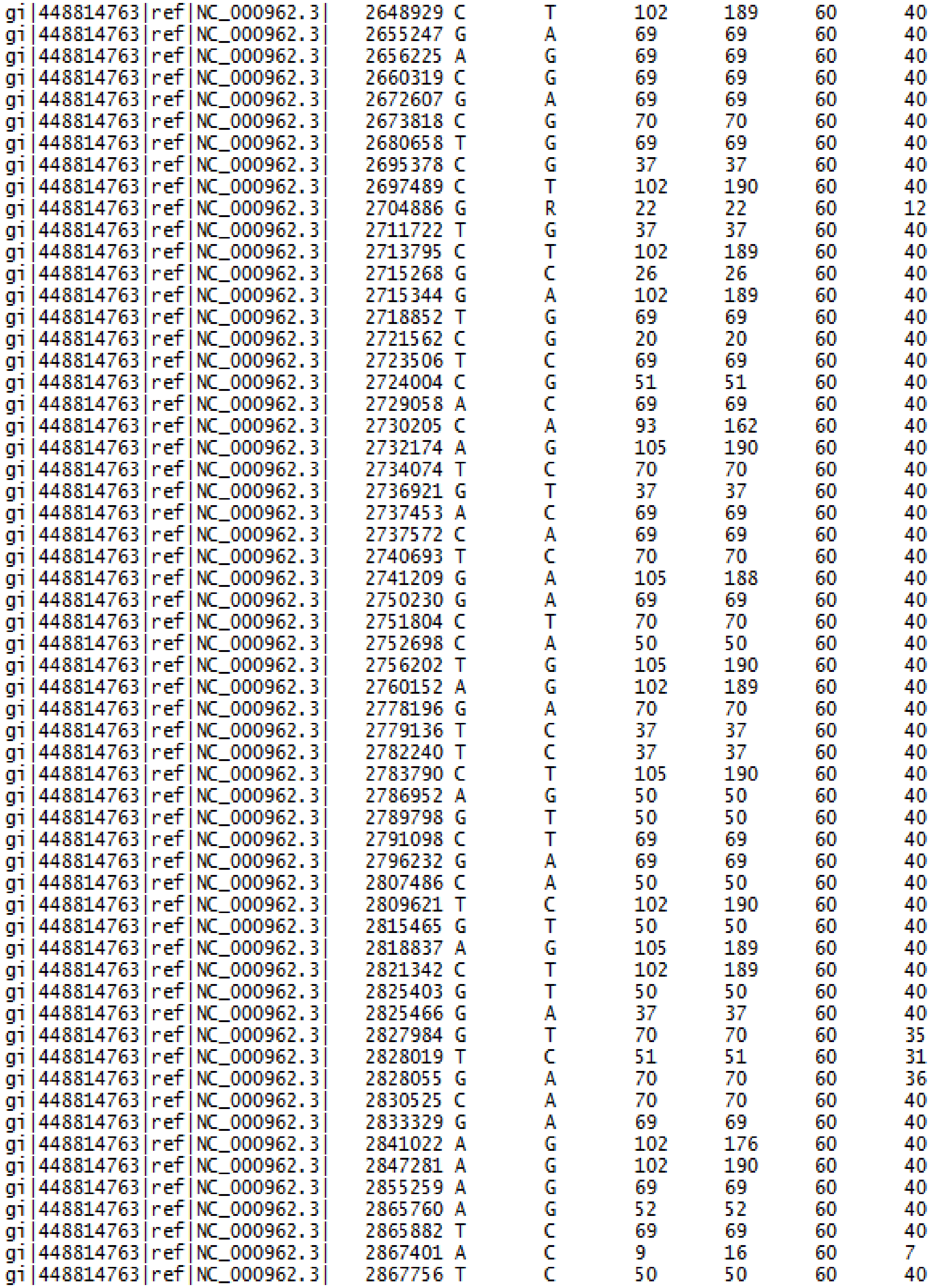

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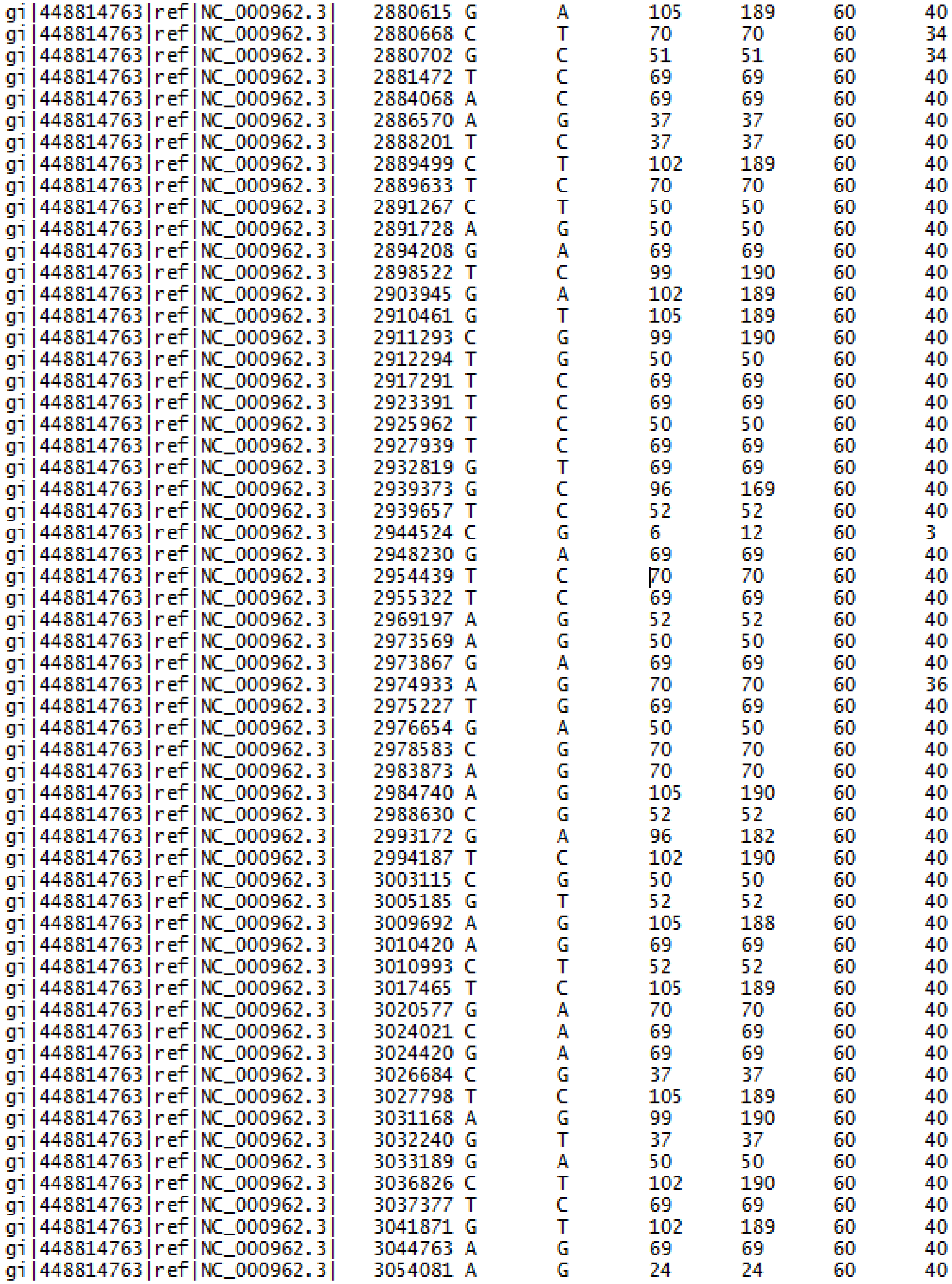

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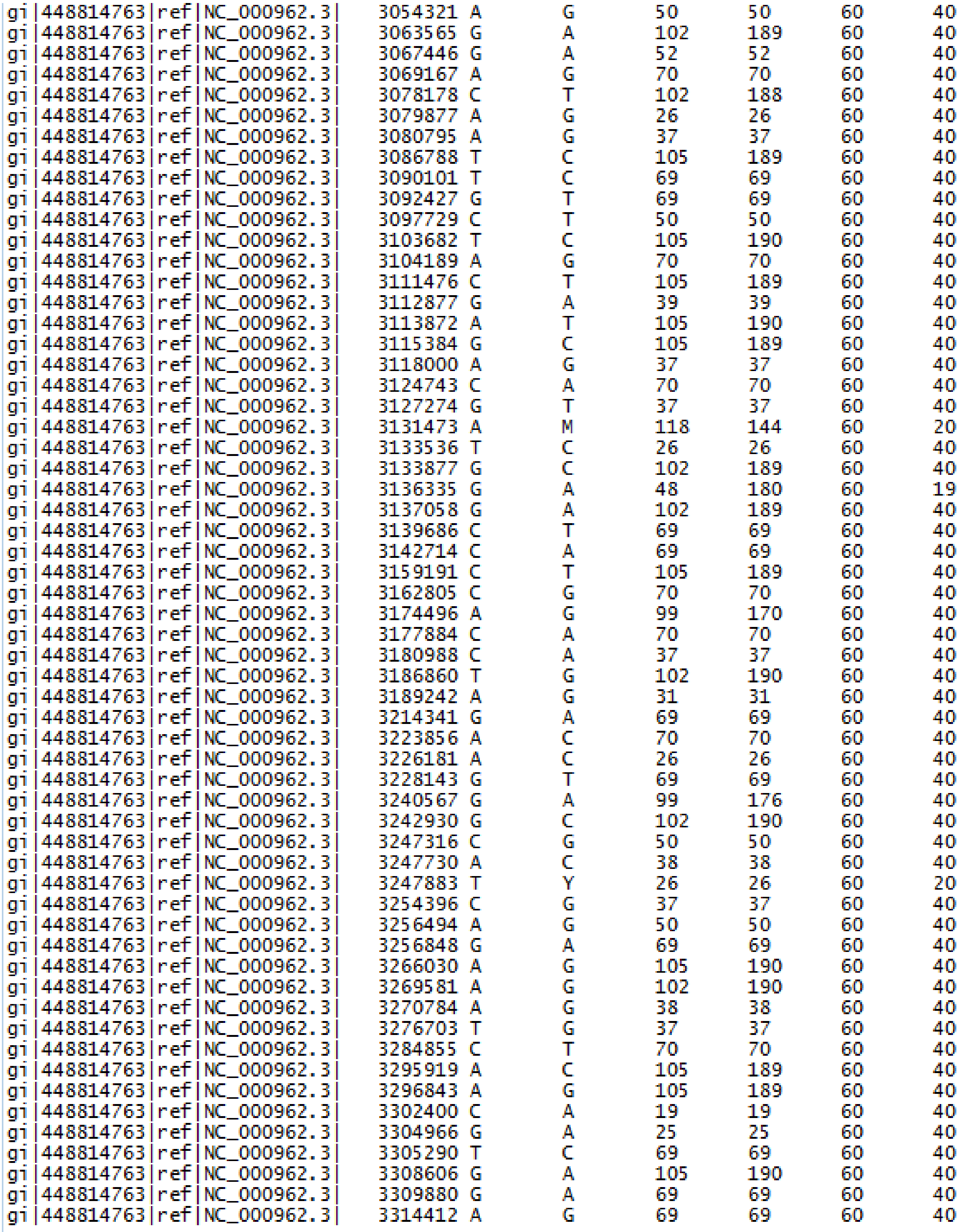

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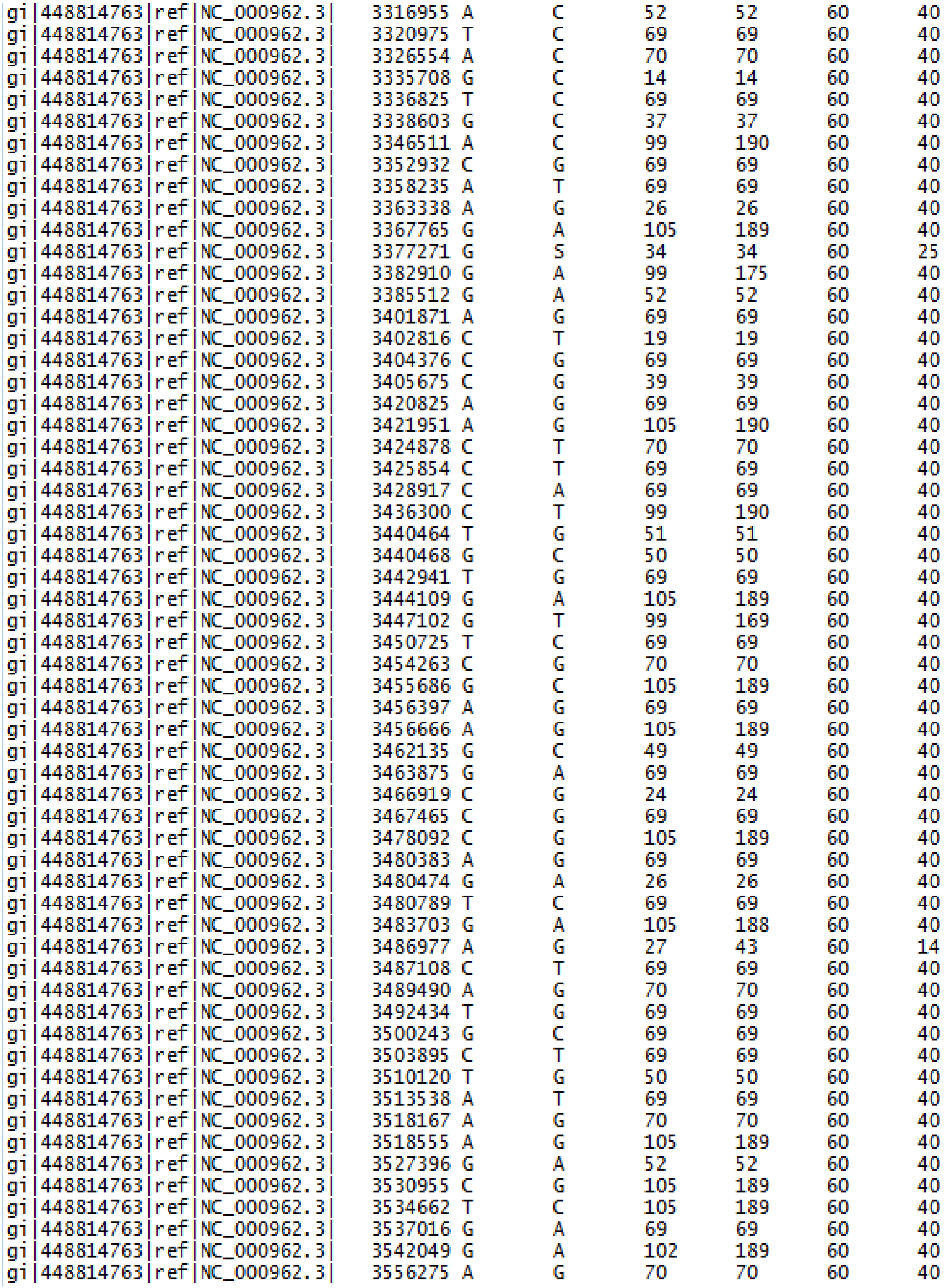

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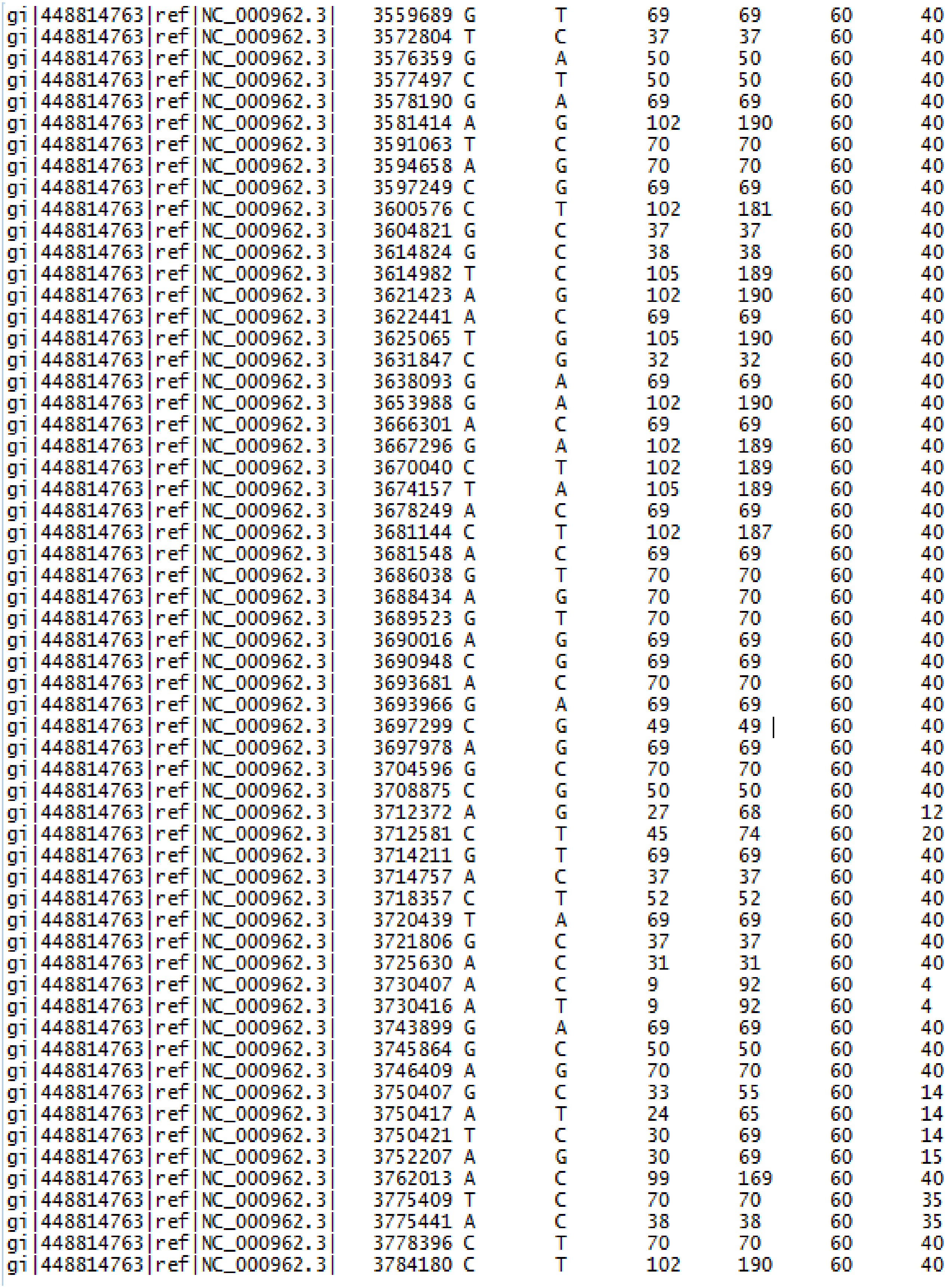

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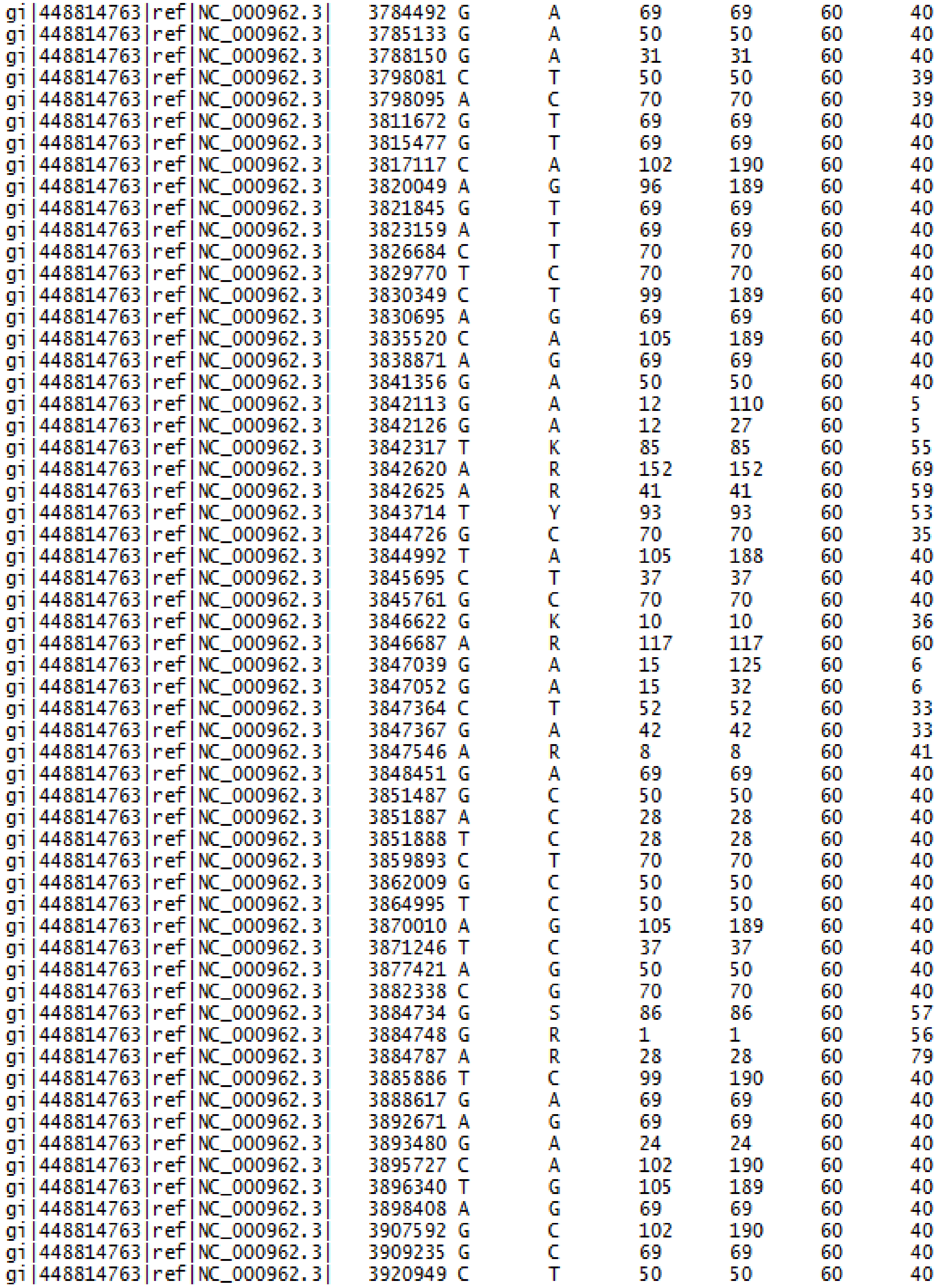

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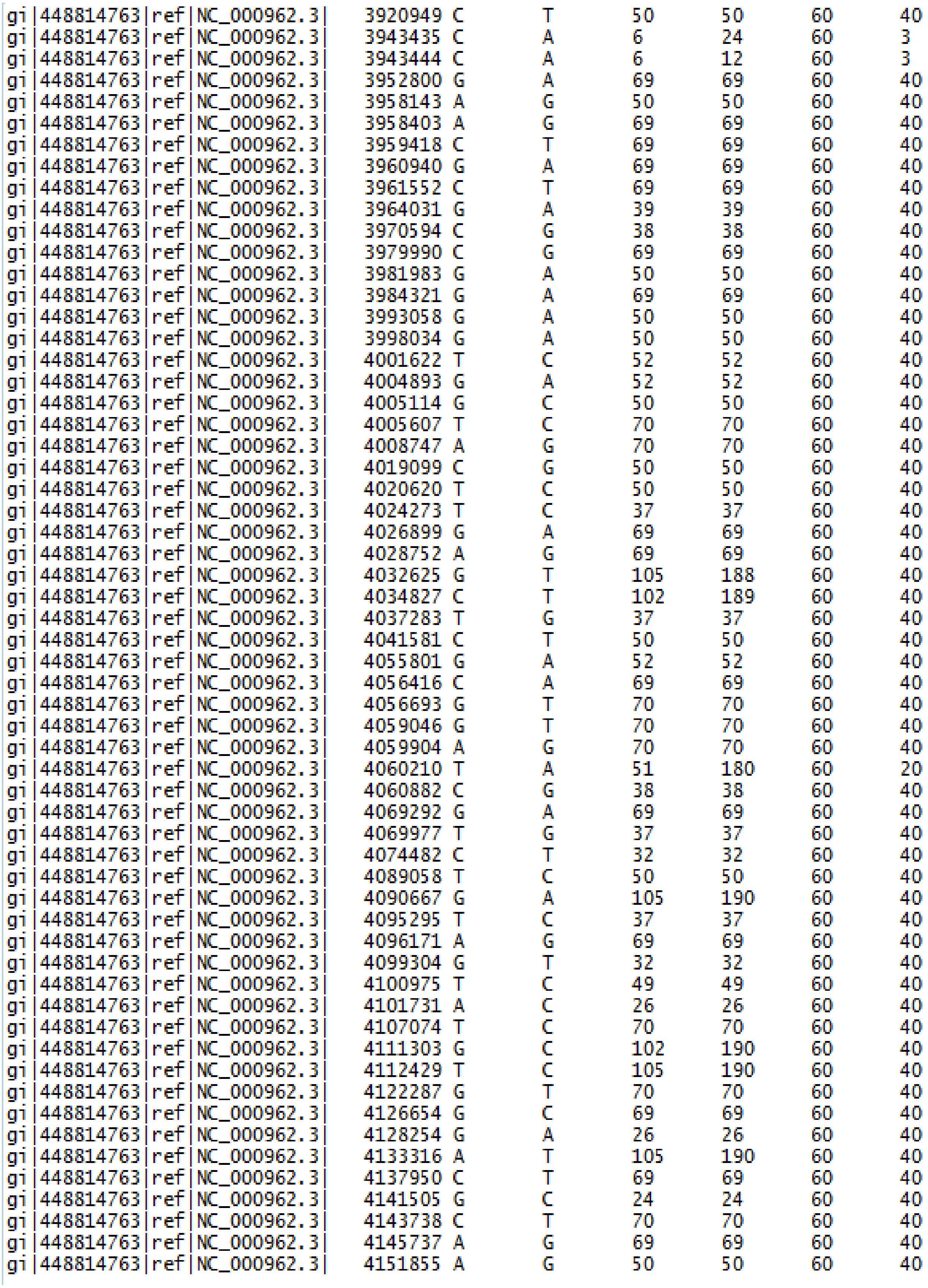

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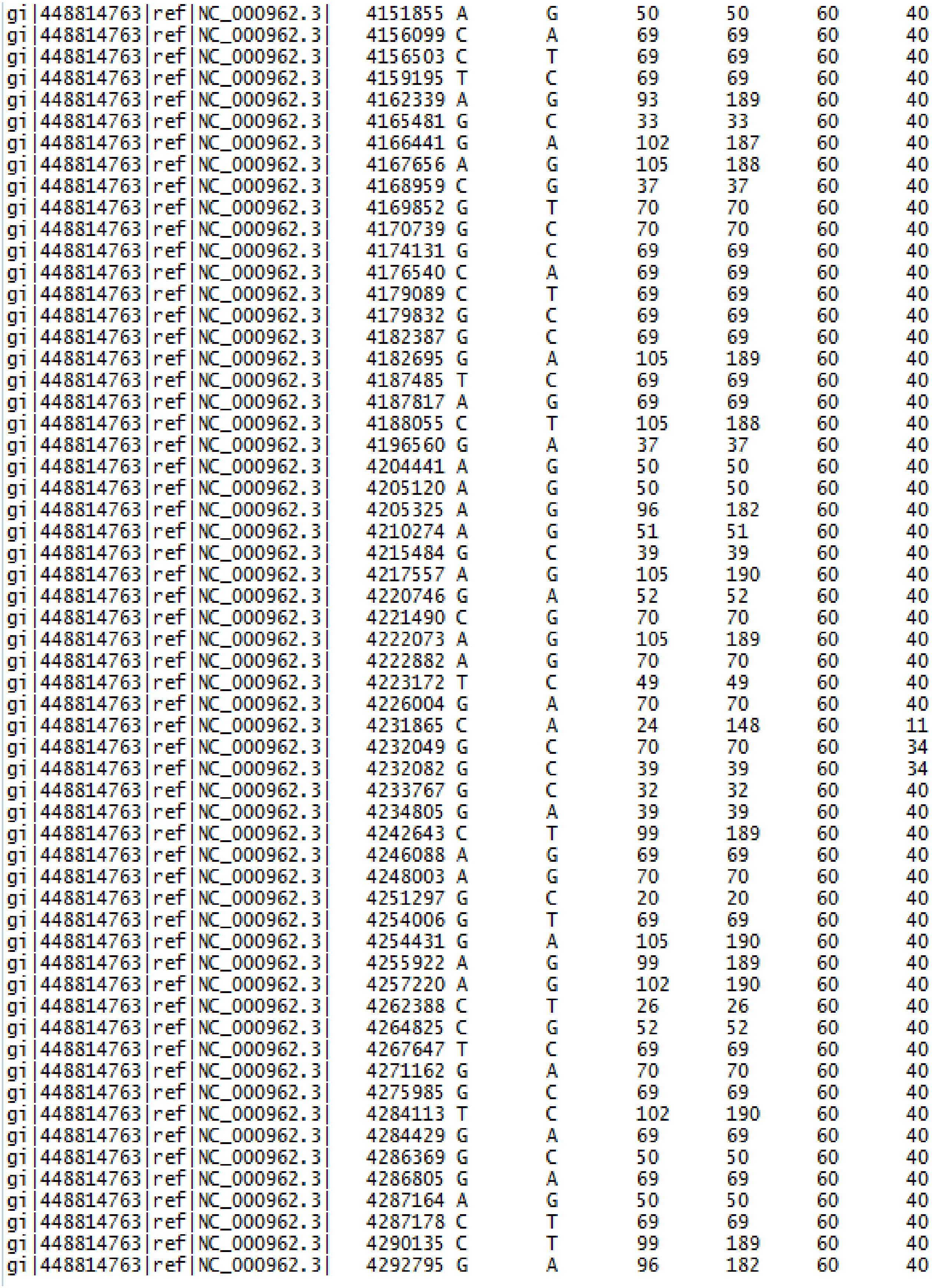

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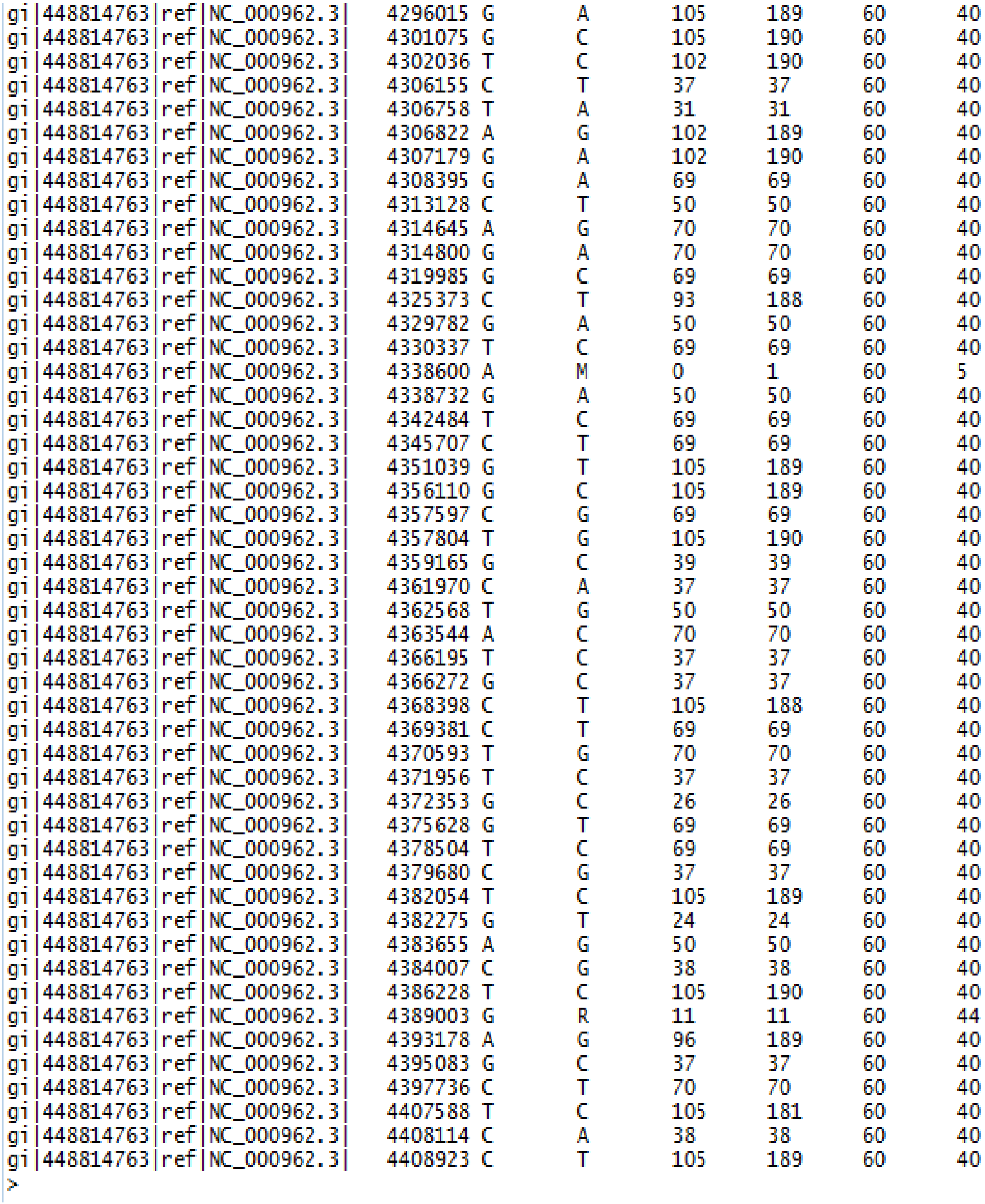

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gi|448814763|ref|NC_000962.3|   4314645 A       G       70      70      60      40
gi|448814763|ref|NC_000962.3|   4314800 G       A       70      70      60      40
gi|448814763|ref|NC_000962.3|   4319985 G       C       69      69      60      40
gi|448814763|ref|NC_000962.3|   4325373 C       T       93      188     60      40
gi|448814763|ref|NC_000962.3|   4329782 G       A       50      50      60      40
gi|448814763|ref|NC_000962.3|   4330337 T       C       69      69      60      40
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gi|448814763|ref|NC_000962.3|   4342484 T       C       69      69      60      40
gi|448814763|ref|NC_000962.3|   4345707 C       T       69      69      60      40
gi|448814763|ref|NC_000962.3|   4351039 G       T       105     189     60      40
gi|448814763|ref|NC_000962.3|   4356110 G       C       105     189     60      40
gi|448814763|ref|NC_000962.3|   4357597 C       G       69      69      60      40
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gi|448814763|ref|NC_000962.3|   4371956 T       C       37      37      60      40
gi|448814763|ref|NC_000962.3|   4372353 G       C       26      26      60      40
gi|448814763|ref|NC_000962.3|   4375628 G       T       69      69      60      40
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gi|448814763|ref|NC_000962.3|   4379680 C       G       37      37      60      40
gi|448814763|ref|NC_000962.3|   4382054 T       C       105     189     60      40
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gi|448814763|ref|NC_000962.3|   4397736 C       T       70      70      60      40
gi|448814763|ref|NC_000962.3|   4407588 T       C       105     181     60      40
gi|448814763|ref|NC_000962.3|   4408114 C       A       38      38      60      40
gi|448814763|ref|NC_000962.3|   4408923 C       T       105     189     60      40
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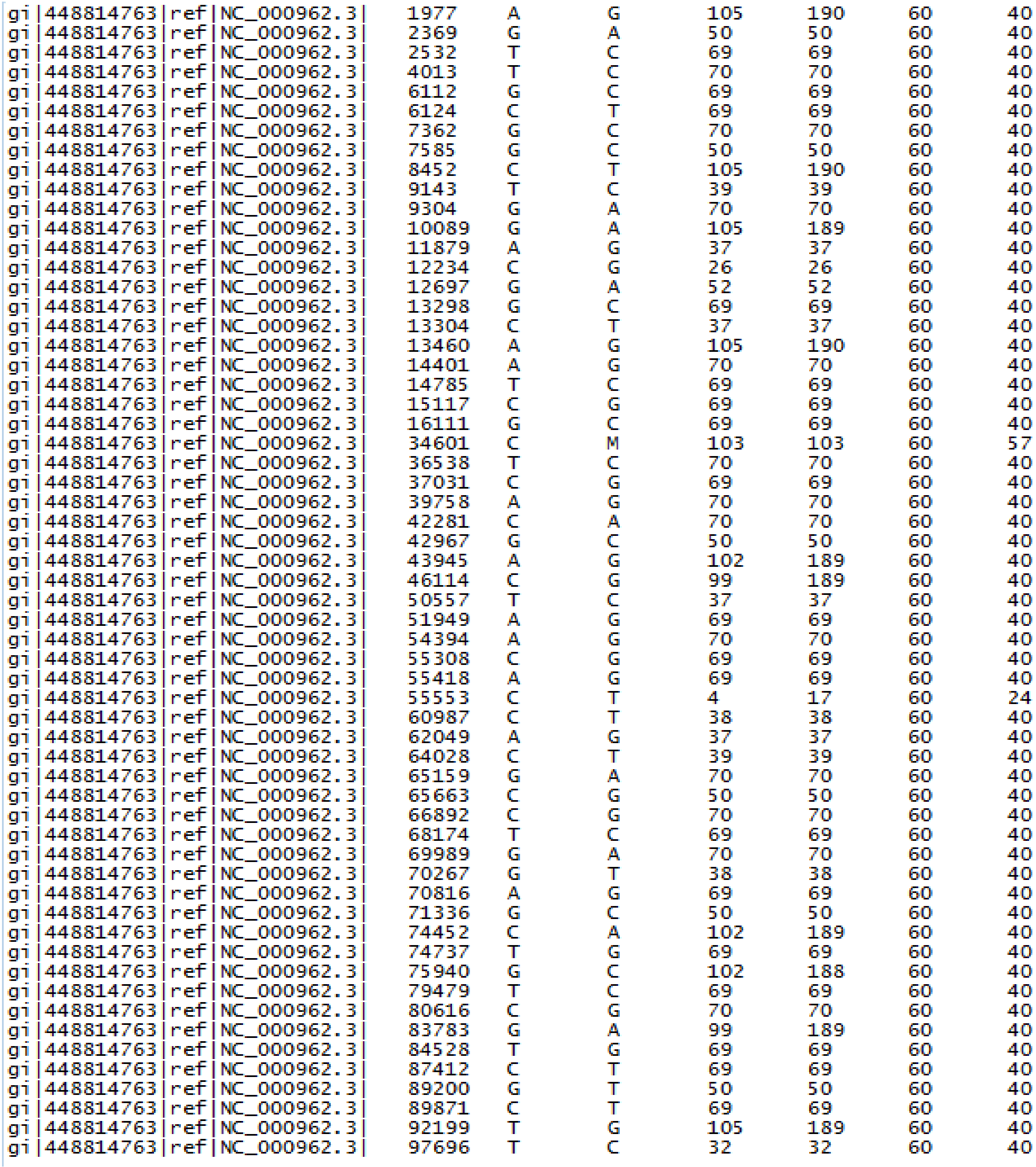
APPENDIX II :-

Alignment of AQQC query strain with *Mycobacterium tuberculosis* strain H37Rv.



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gi|448814763|ref|NC_000962.3|	7585	G	C	50	50	60	40
gi|448814763|ref|NC_000962.3|	8452	C	T	105	190	60	40
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gi|448814763|ref|NC_000962.3|	12234	C	G	26	26	60	40
gi|448814763|ref|NC_000962.3|	12697	G	A	52	52	60	40
gi|448814763|ref|NC_000962.3|	13298	G	C	69	69	60	40
gi|448814763|ref|NC_000962.3|	13304	C	T	37	37	60	40
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gi|448814763|ref|NC_000962.3|	14785	T	C	69	69	60	40
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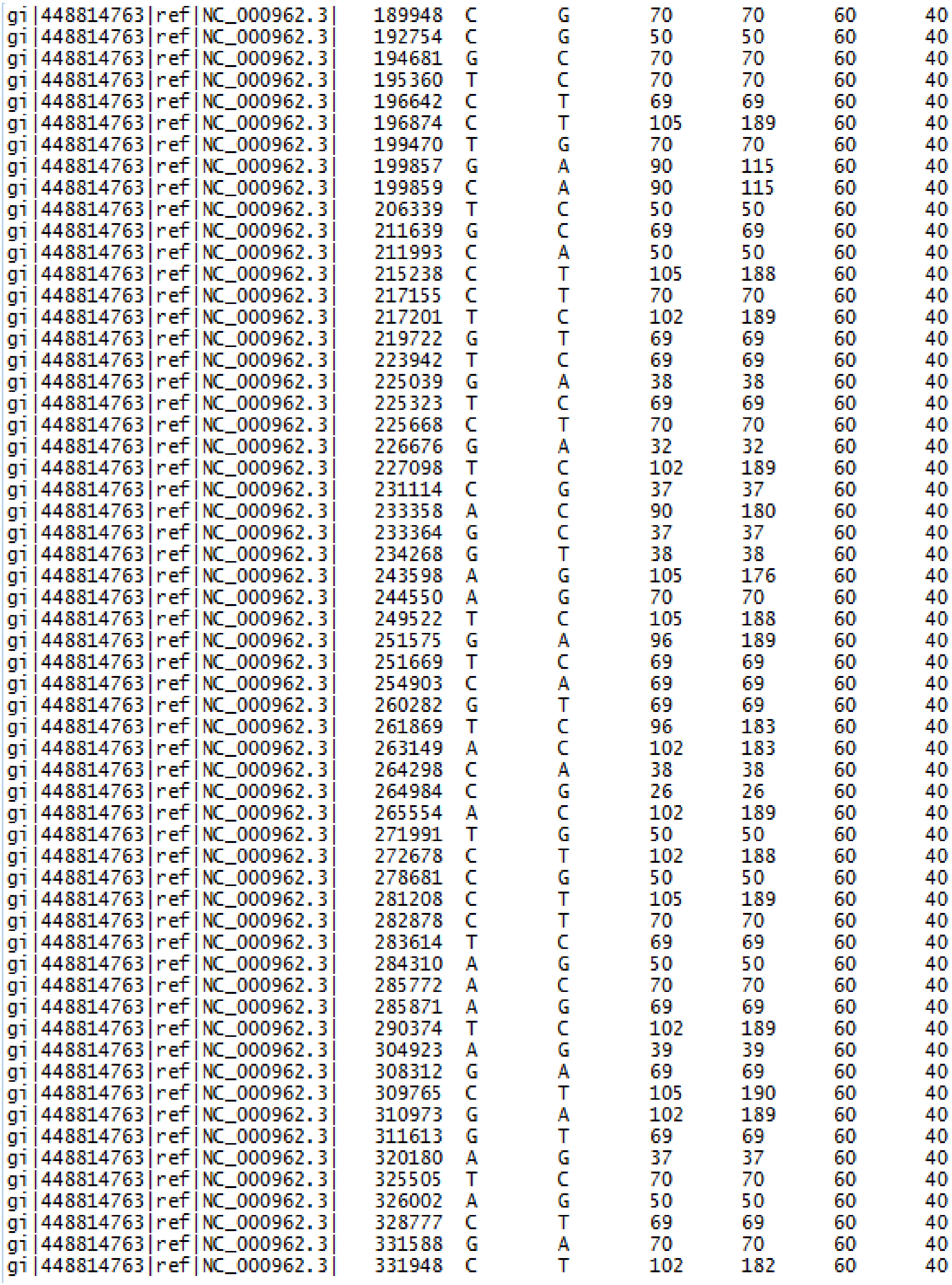

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gi|448814763|ref|NC_000962.3|   195360  T       C       70      70      60      40
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gi|448814763|ref|NC_000962.3|   206339  T       C       50      50      60      40
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gi|448814763|ref|NC_000962.3|   217155  C       T       70      70      60      40
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gi|448814763|ref|NC_000962.3|   219722  G       T       69      69      60      40
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gi|448814763|ref|NC_000962.3|   271991  T       G       50      50      60      40
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gi|448814763|ref|NC_000962.3|   281208  C       T       105     189     60      40
gi|448814763|ref|NC_000962.3|   282878  C       T       70      70      60      40
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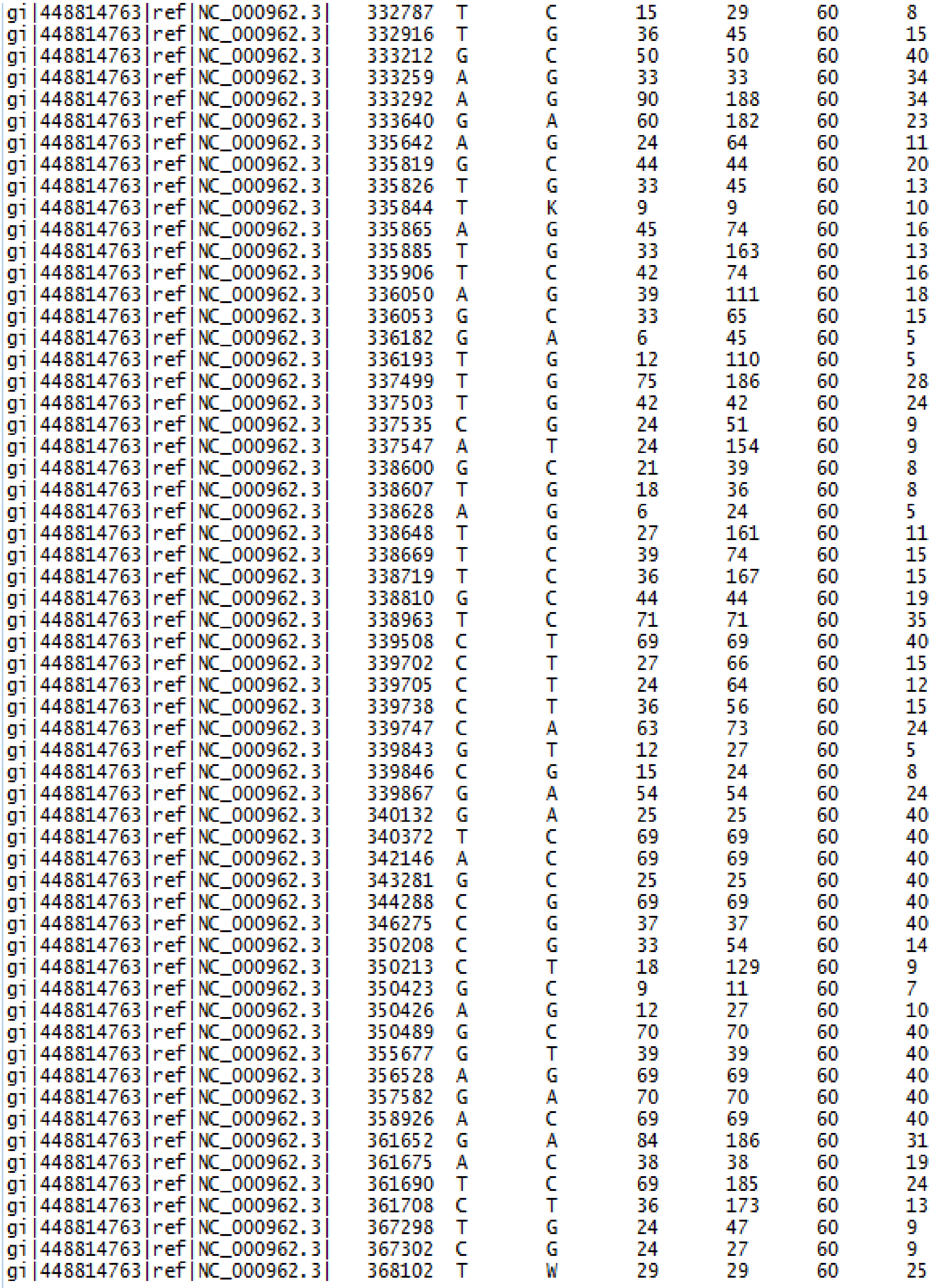

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gi|448814763|ref|NC_000962.3|    333640  G    A    60   182  60   23
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gi|448814763|ref|NC_000962.3|    335819  G    C    44   44   60   20
gi|448814763|ref|NC_000962.3|    335826  T    G    33   45   60   13
gi|448814763|ref|NC_000962.3|    335844  T    K    9    9    60   10
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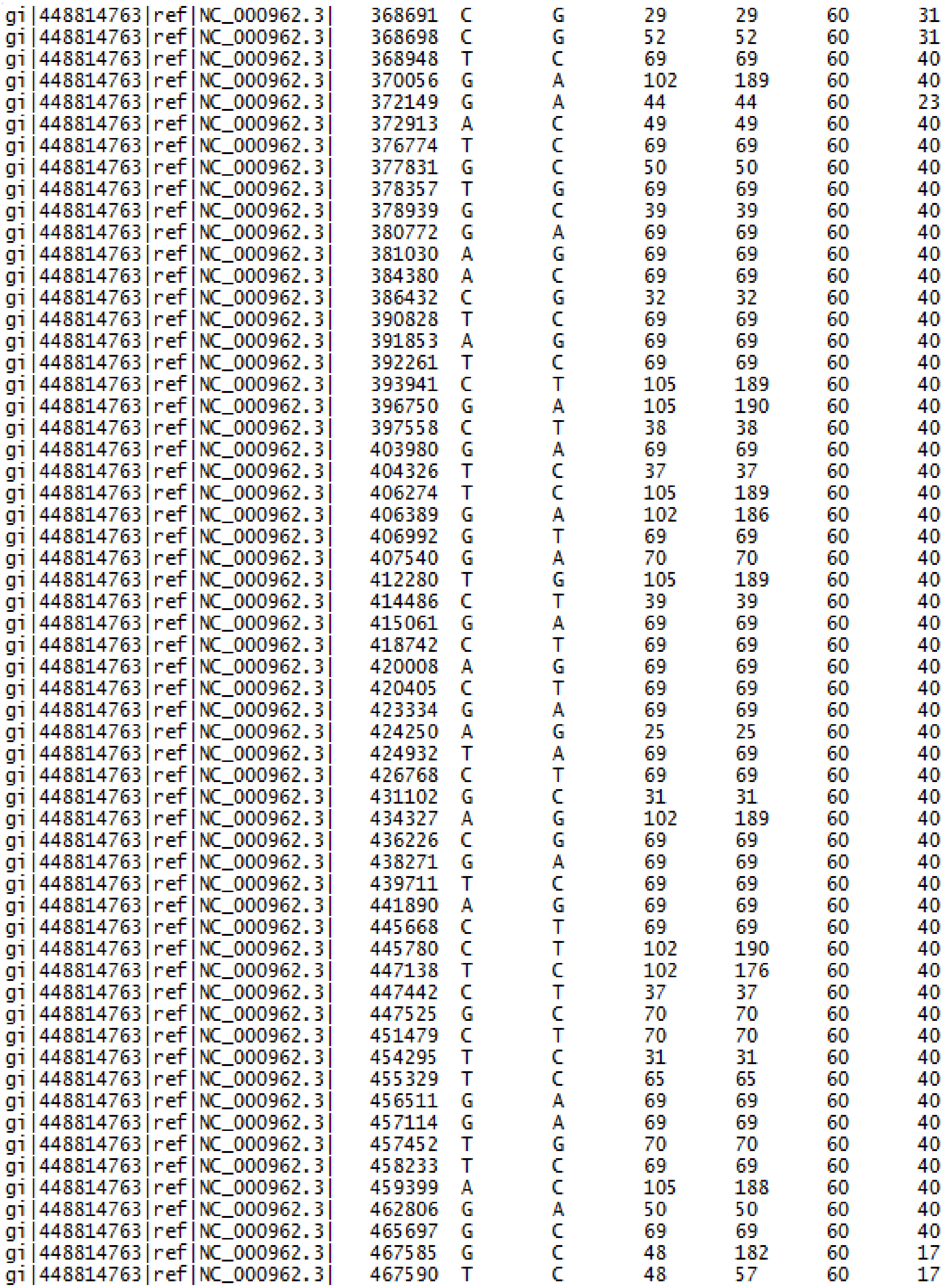

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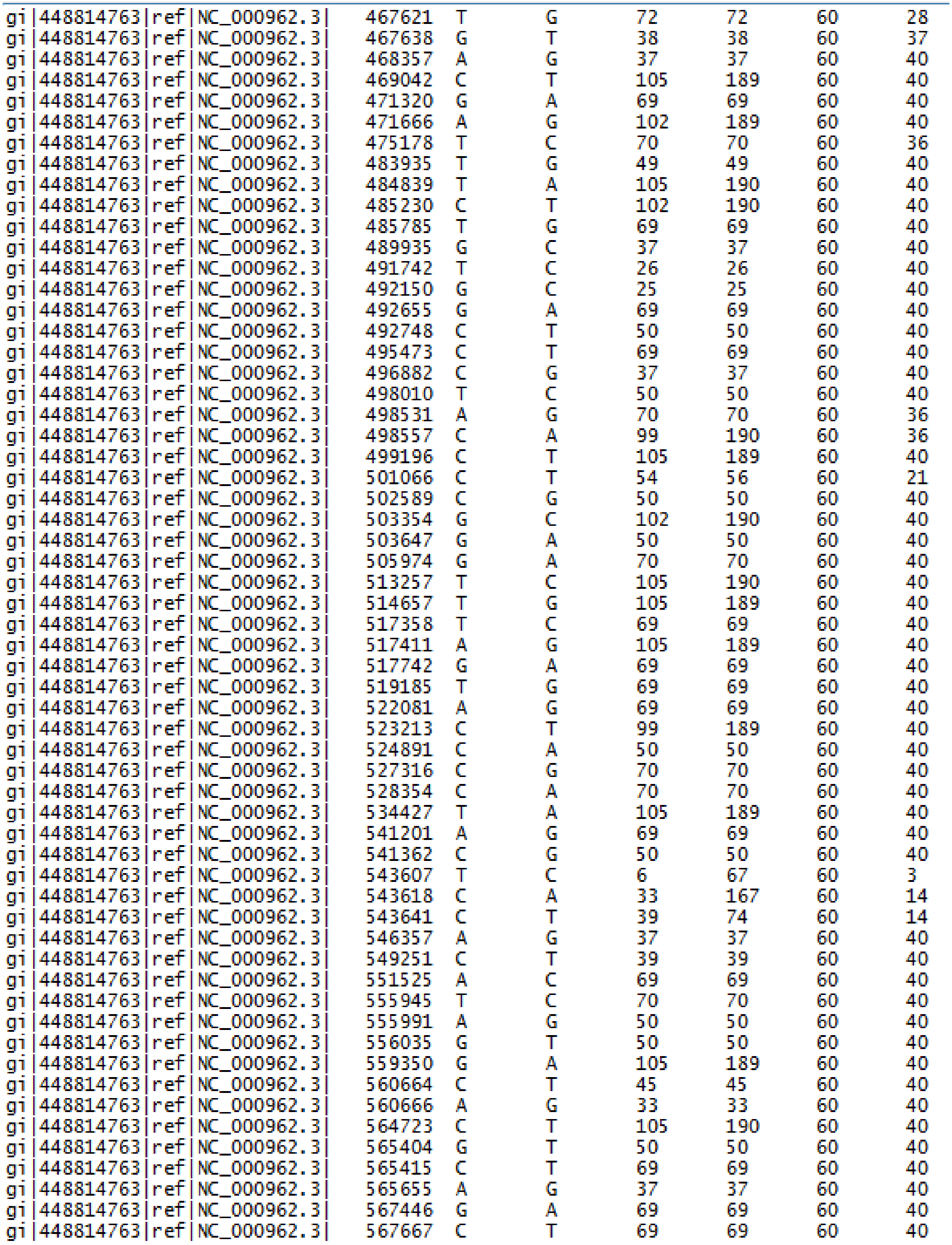

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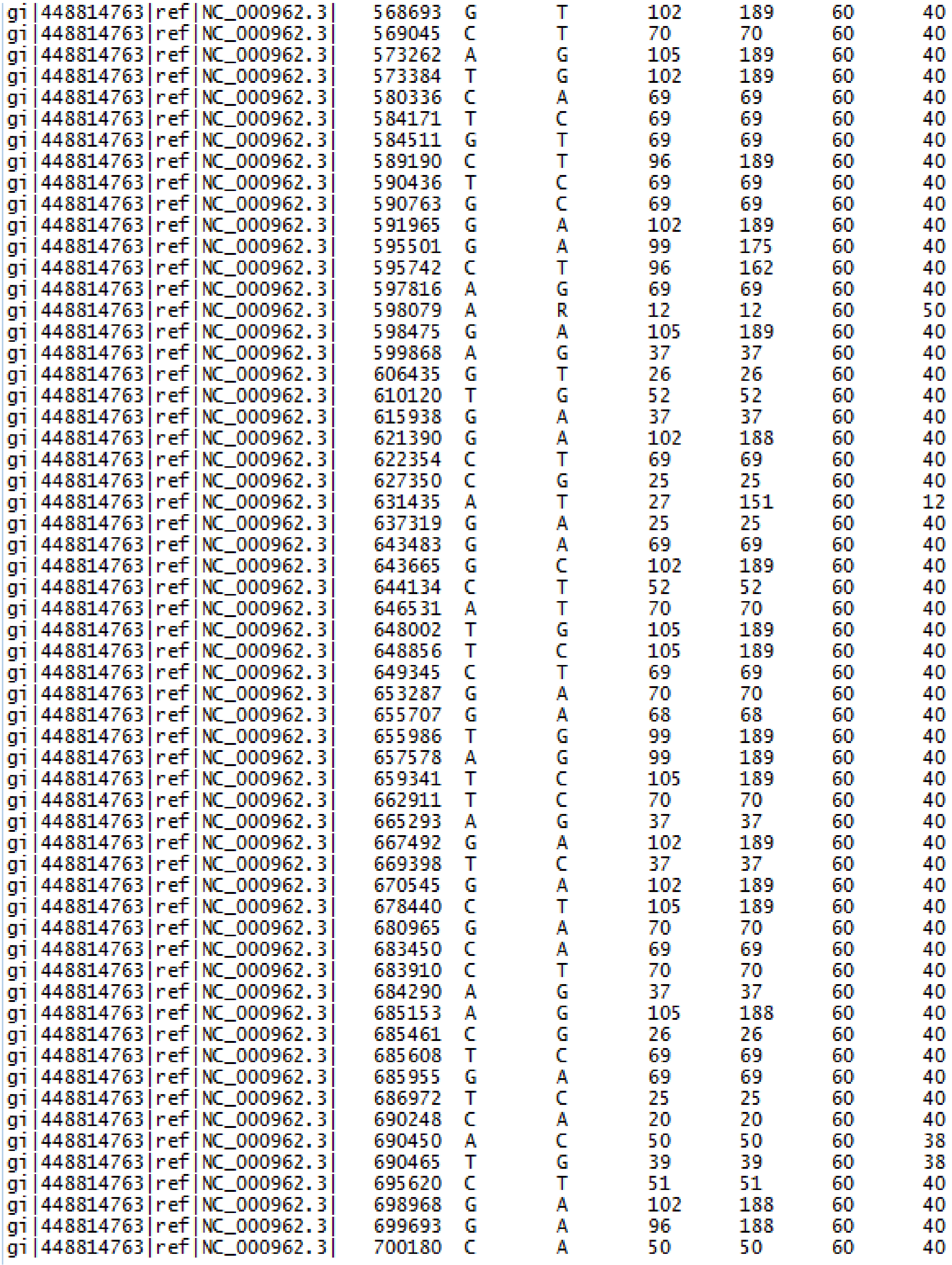

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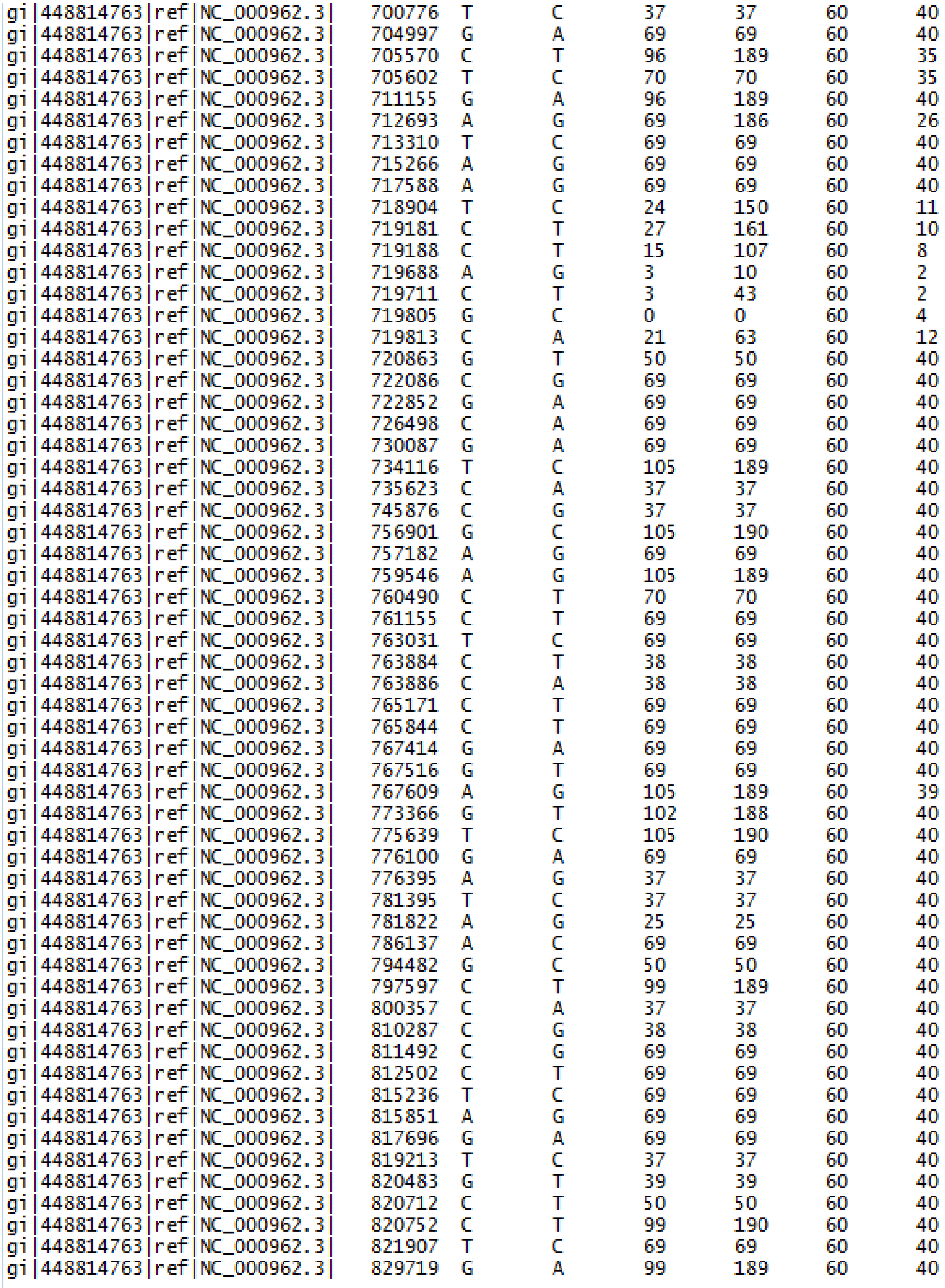

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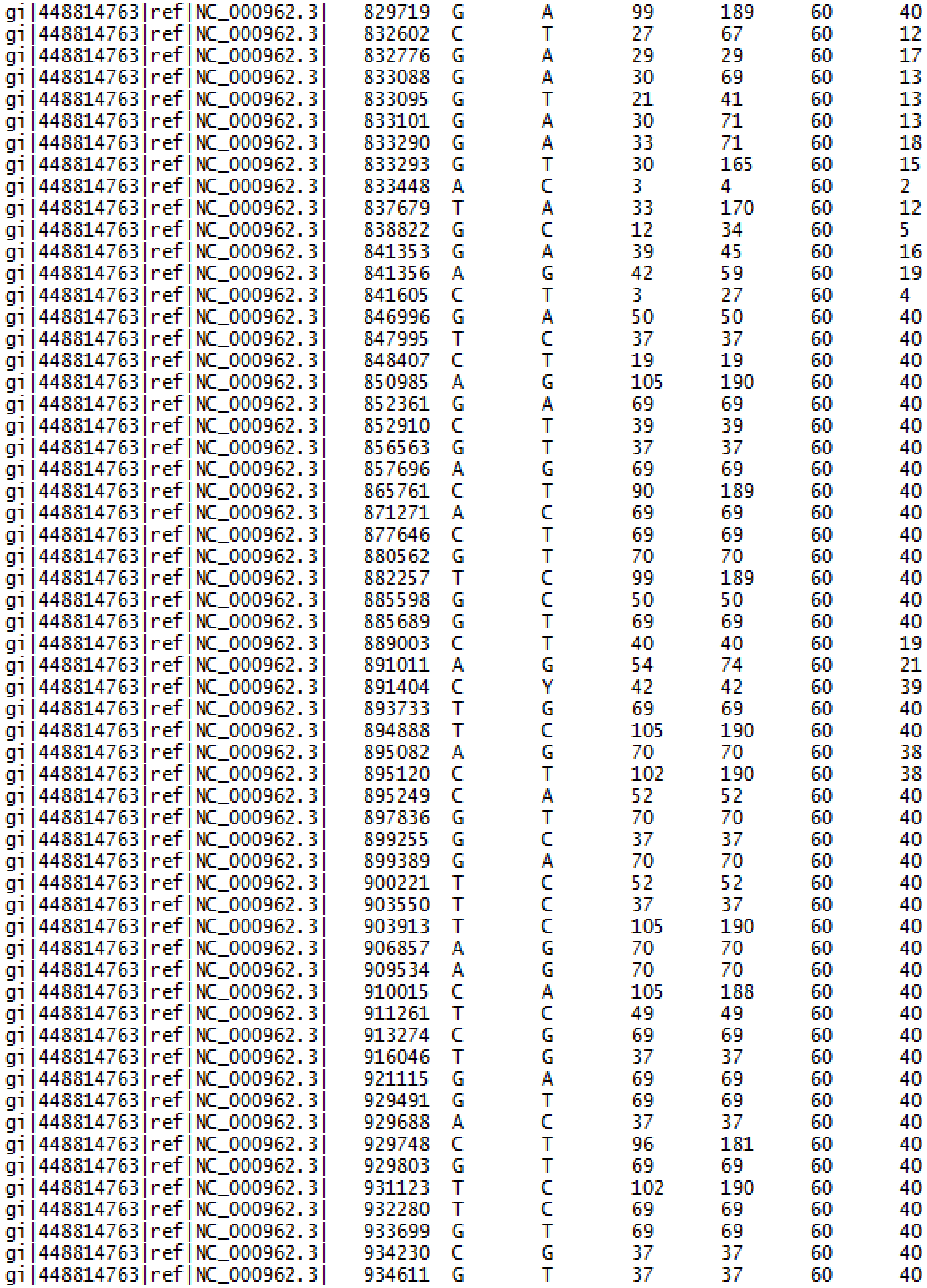

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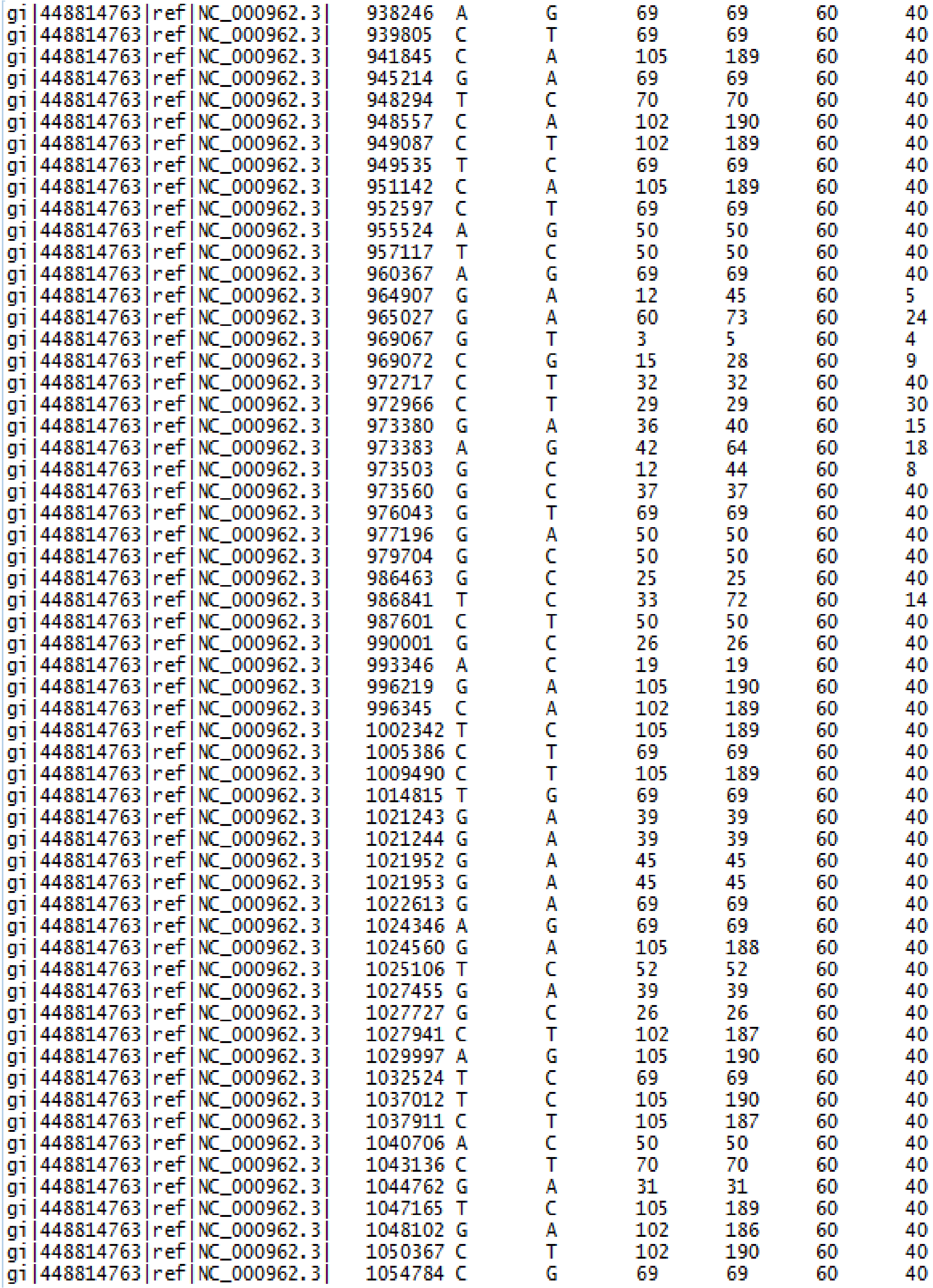

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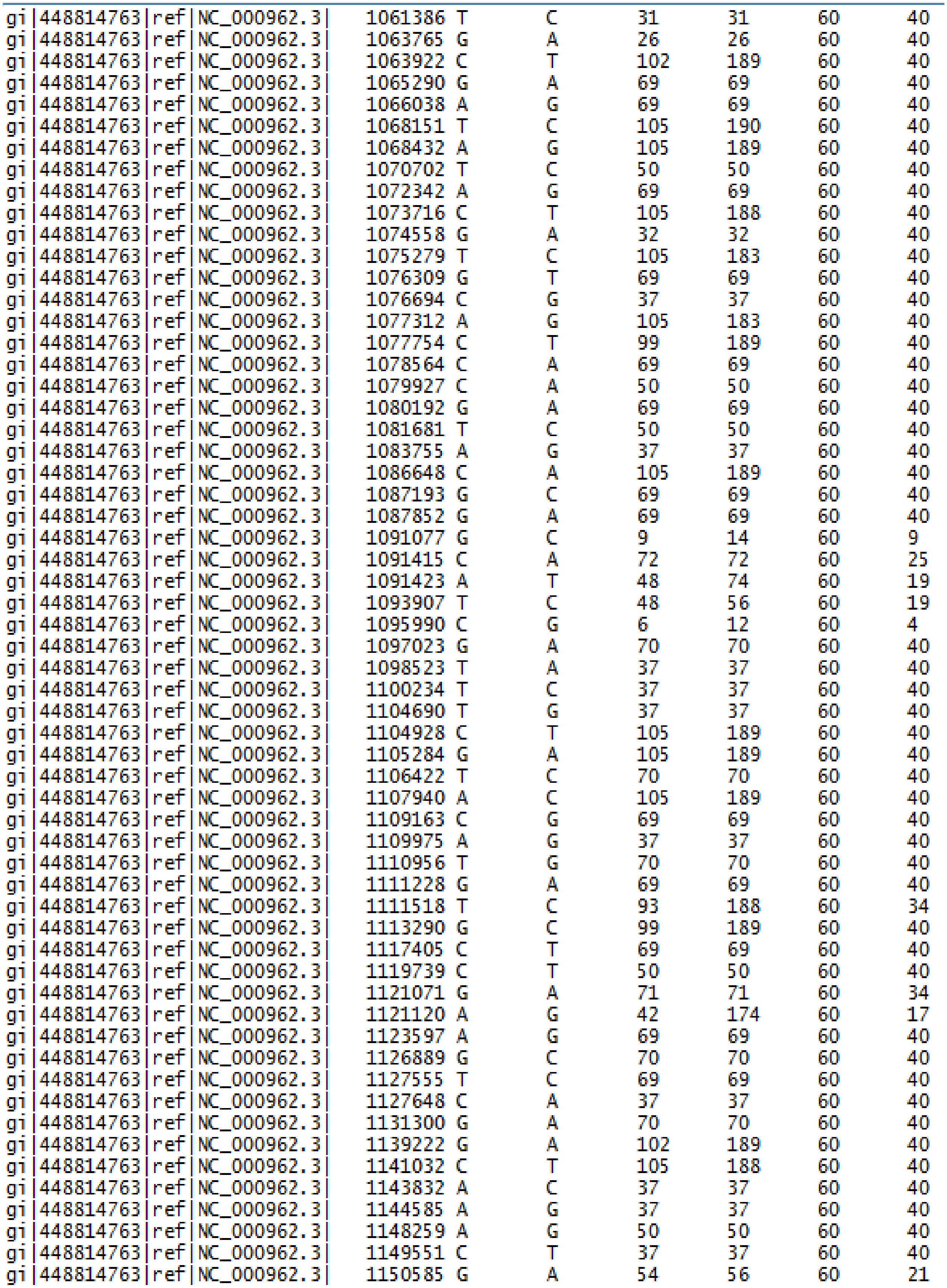

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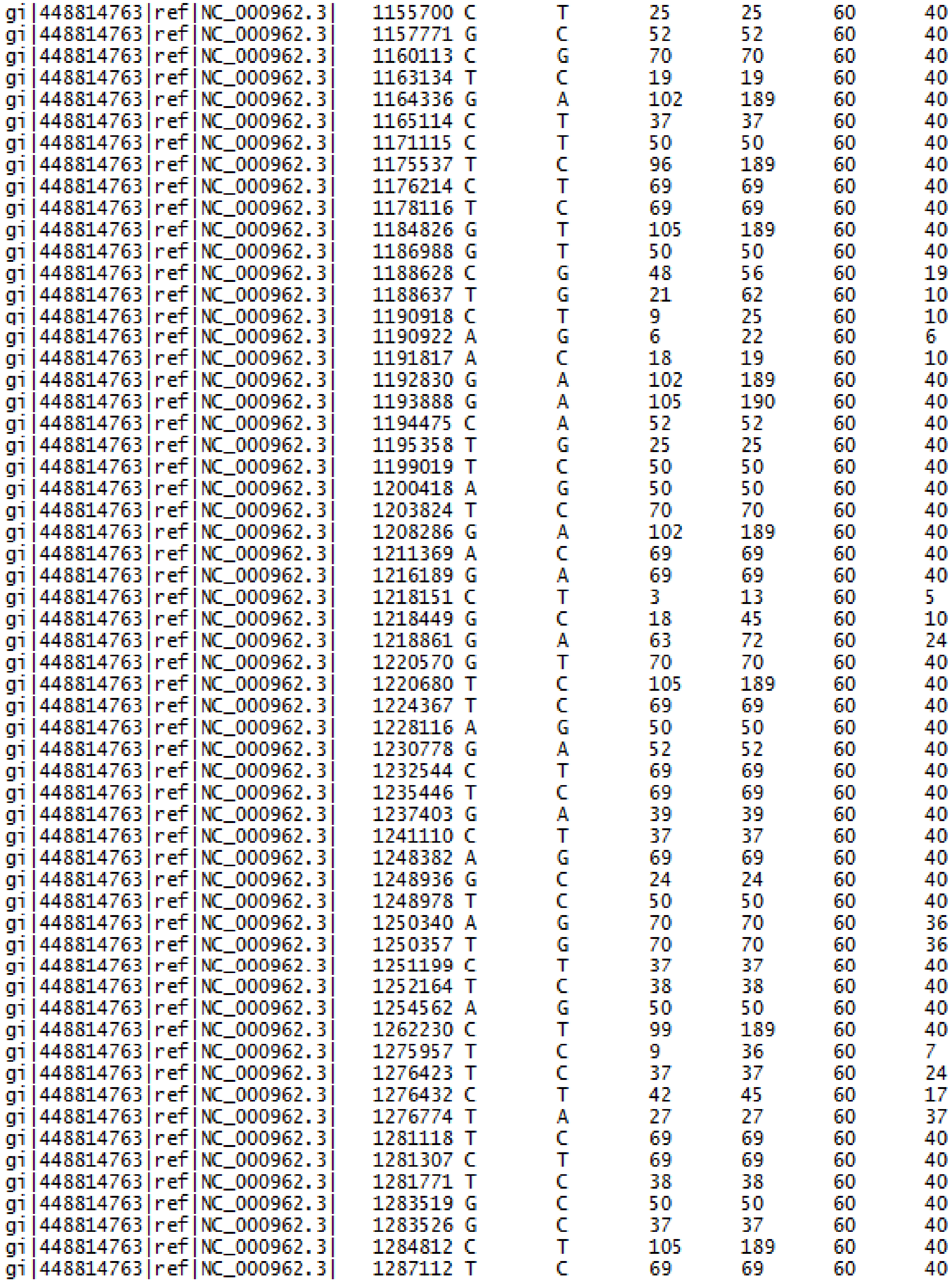

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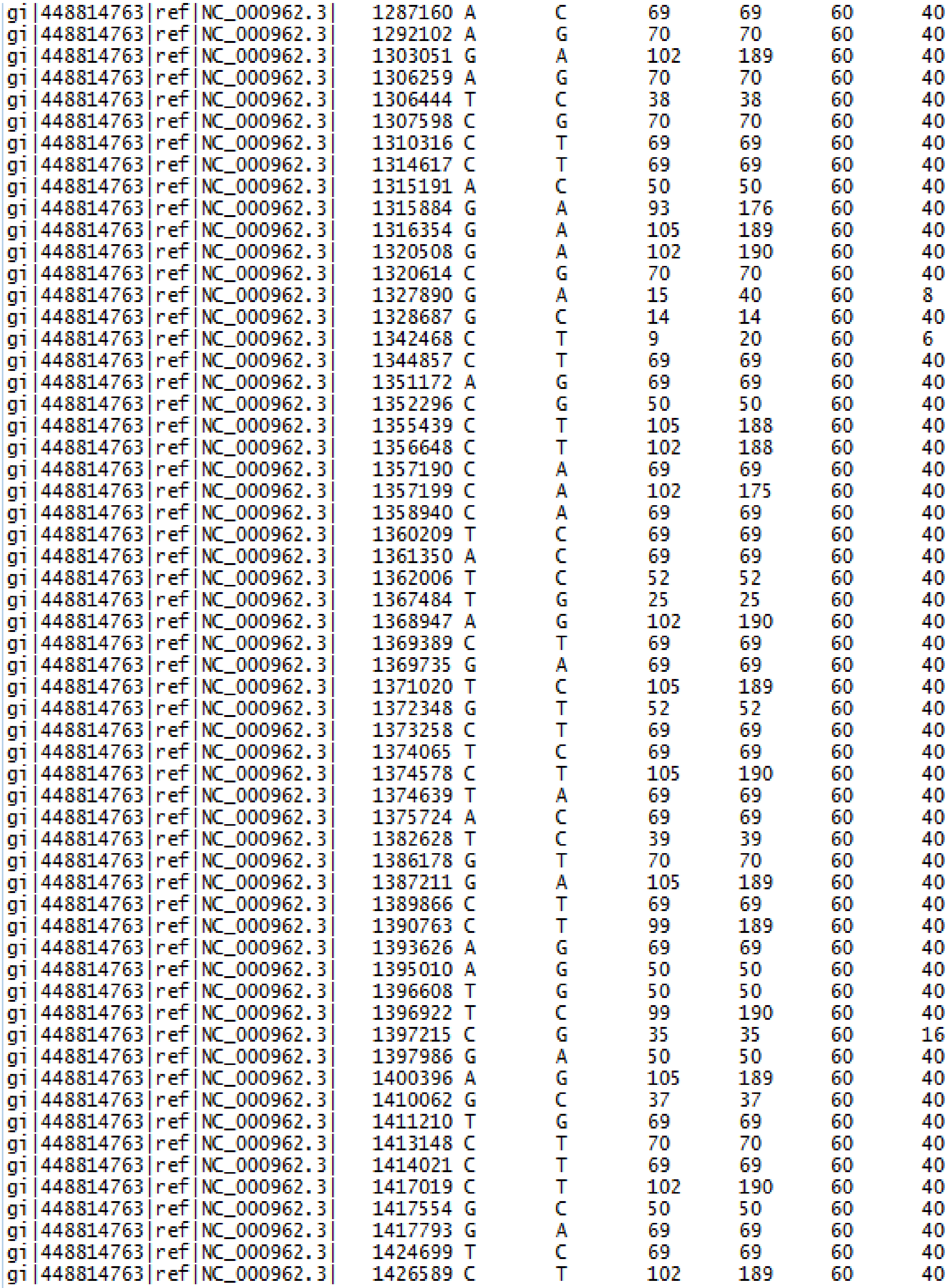

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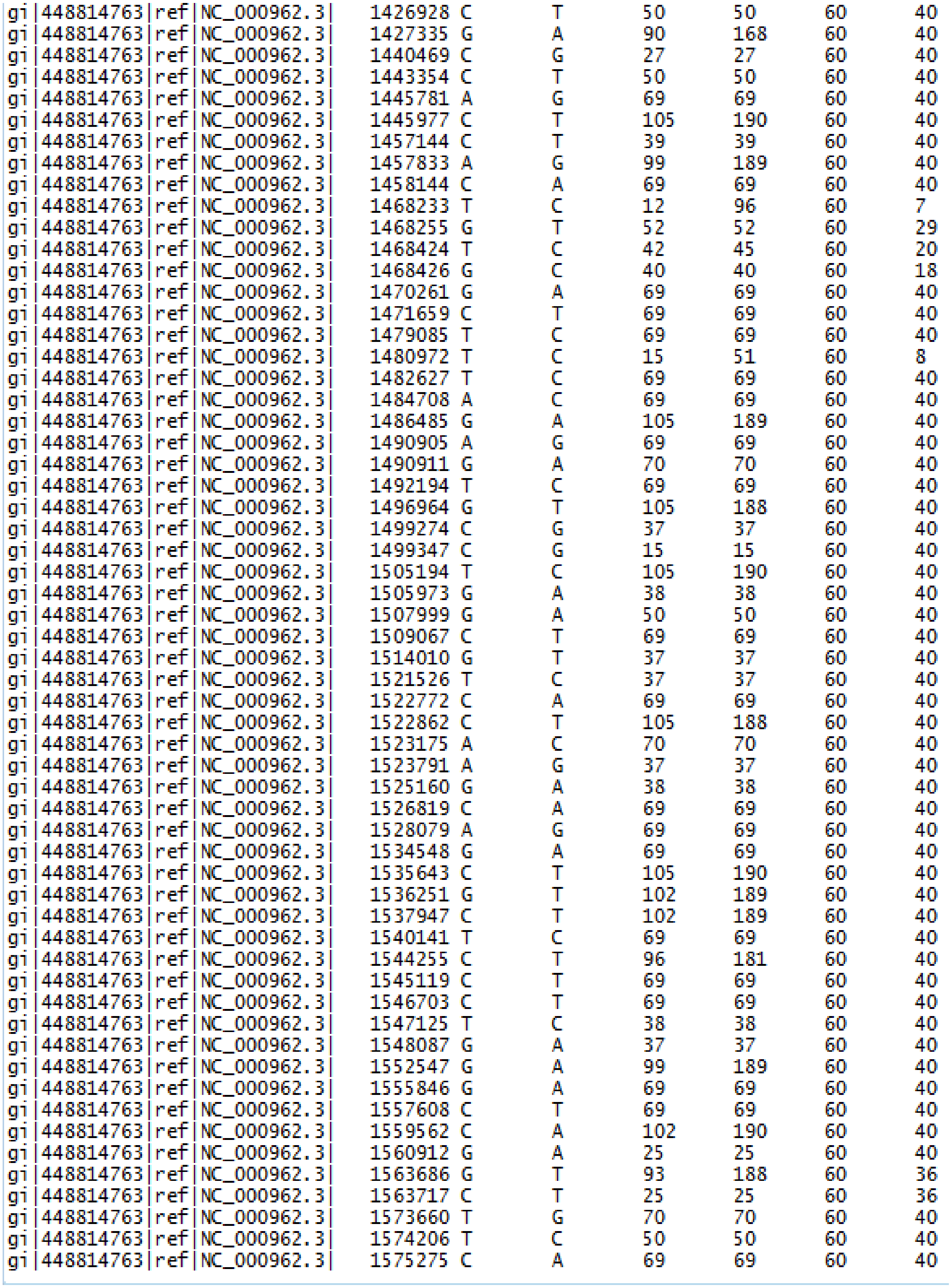

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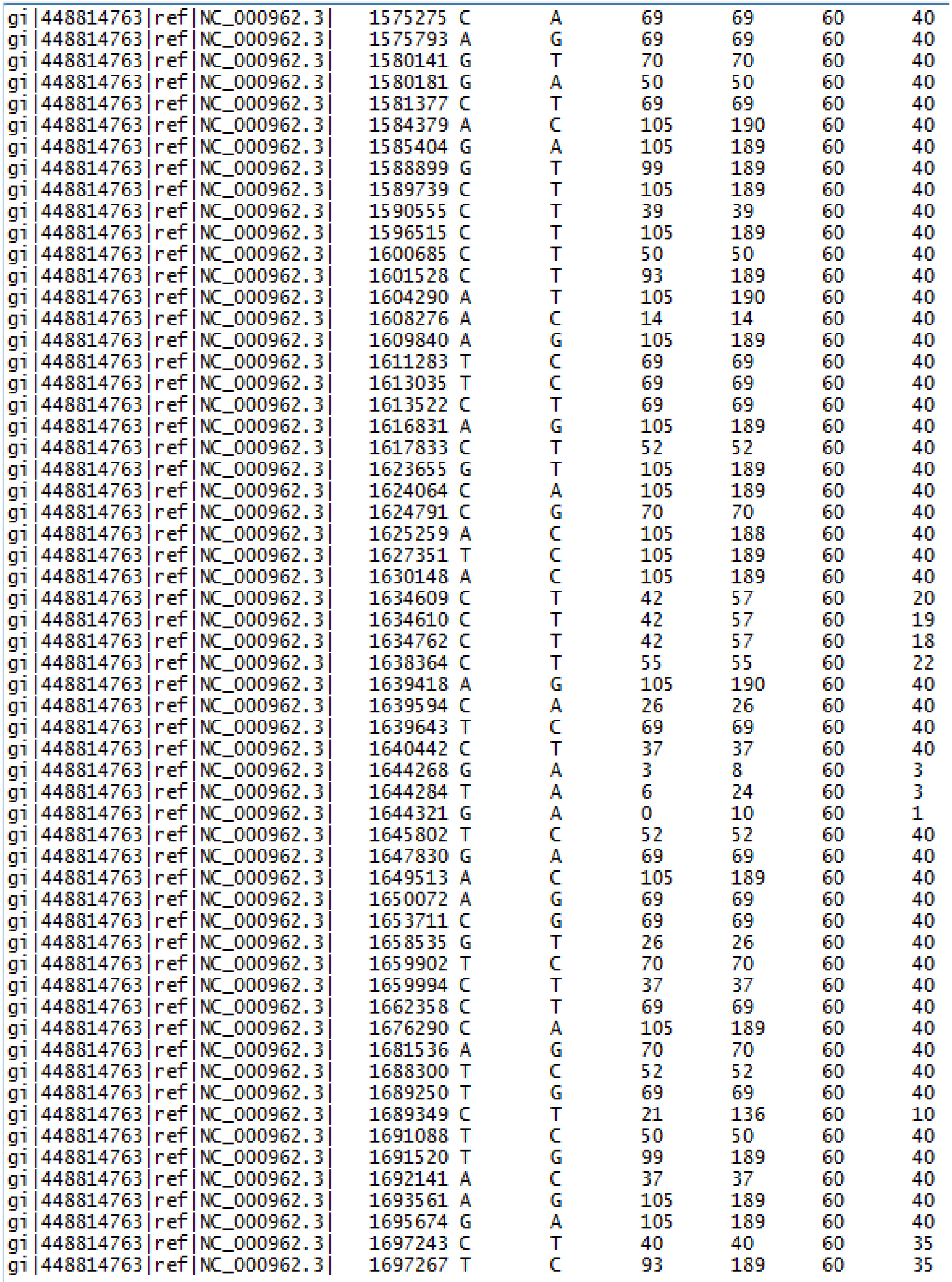

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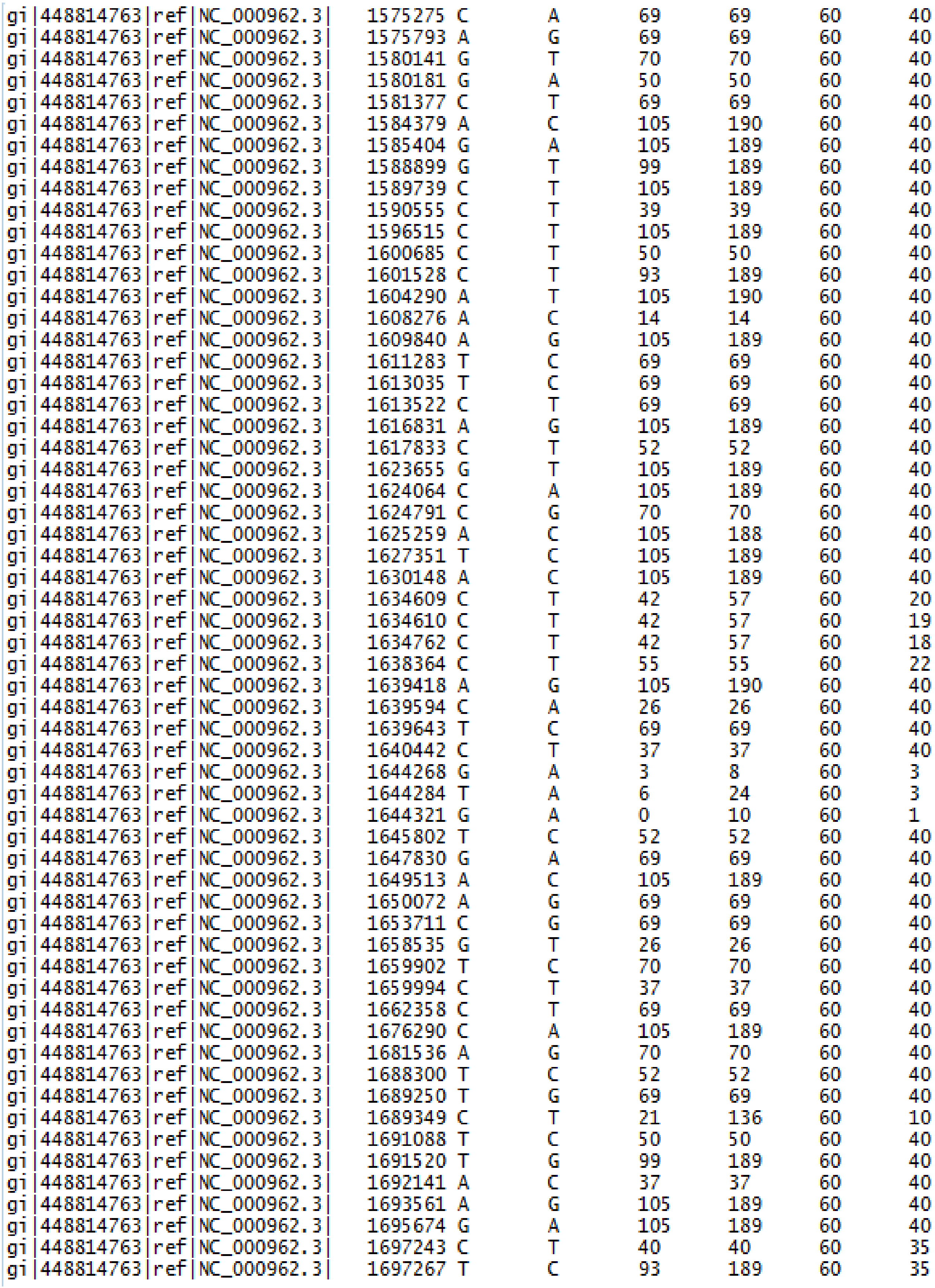

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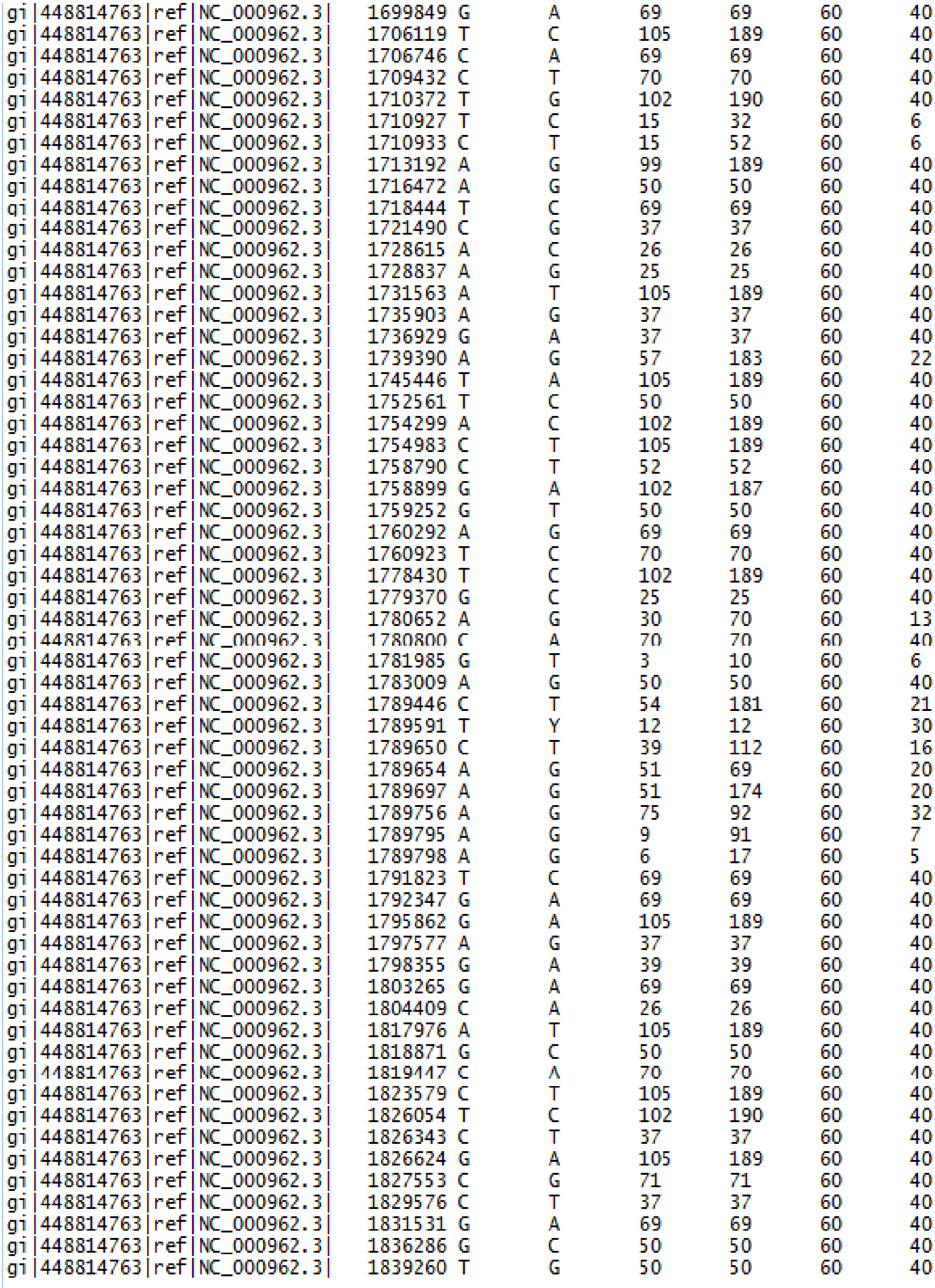

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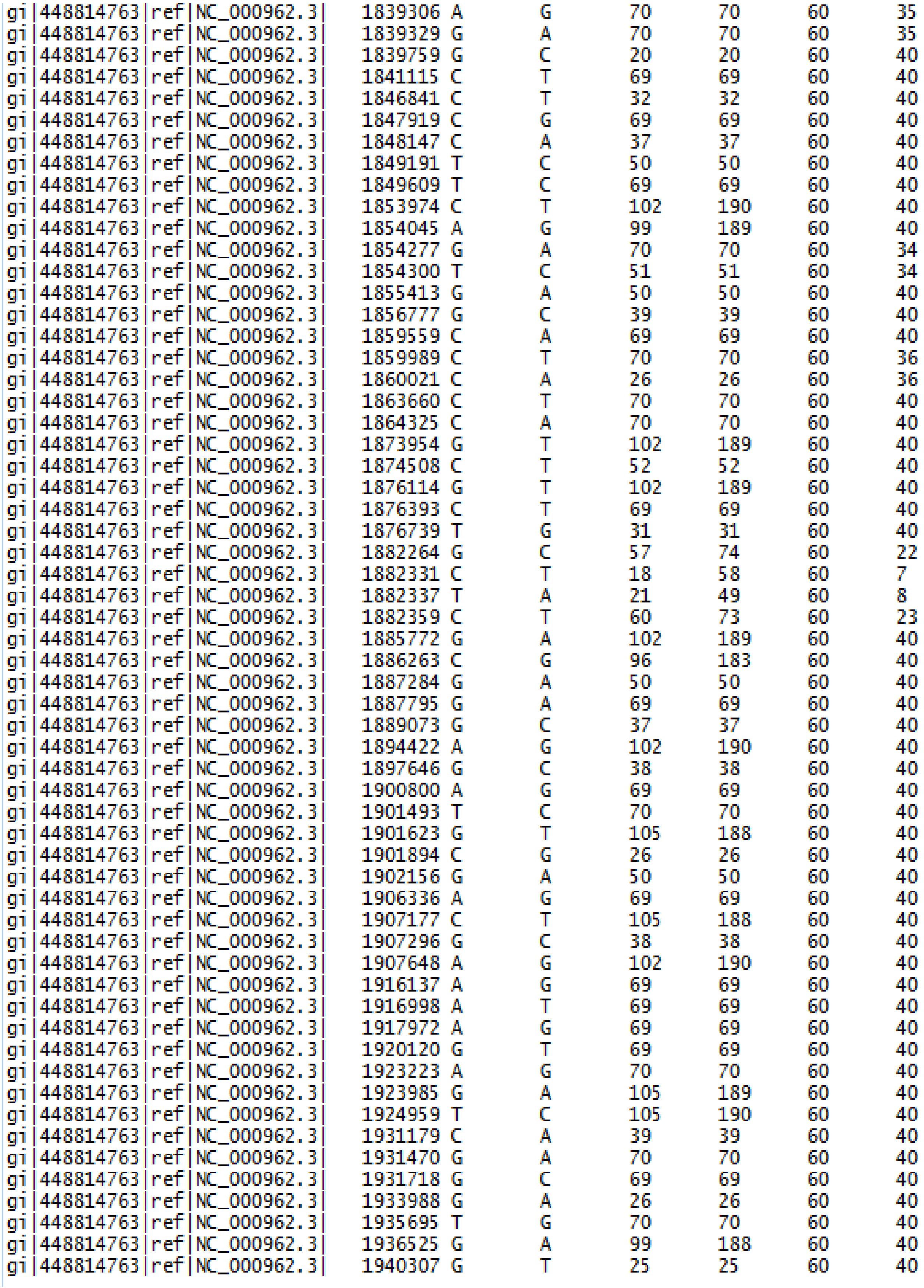

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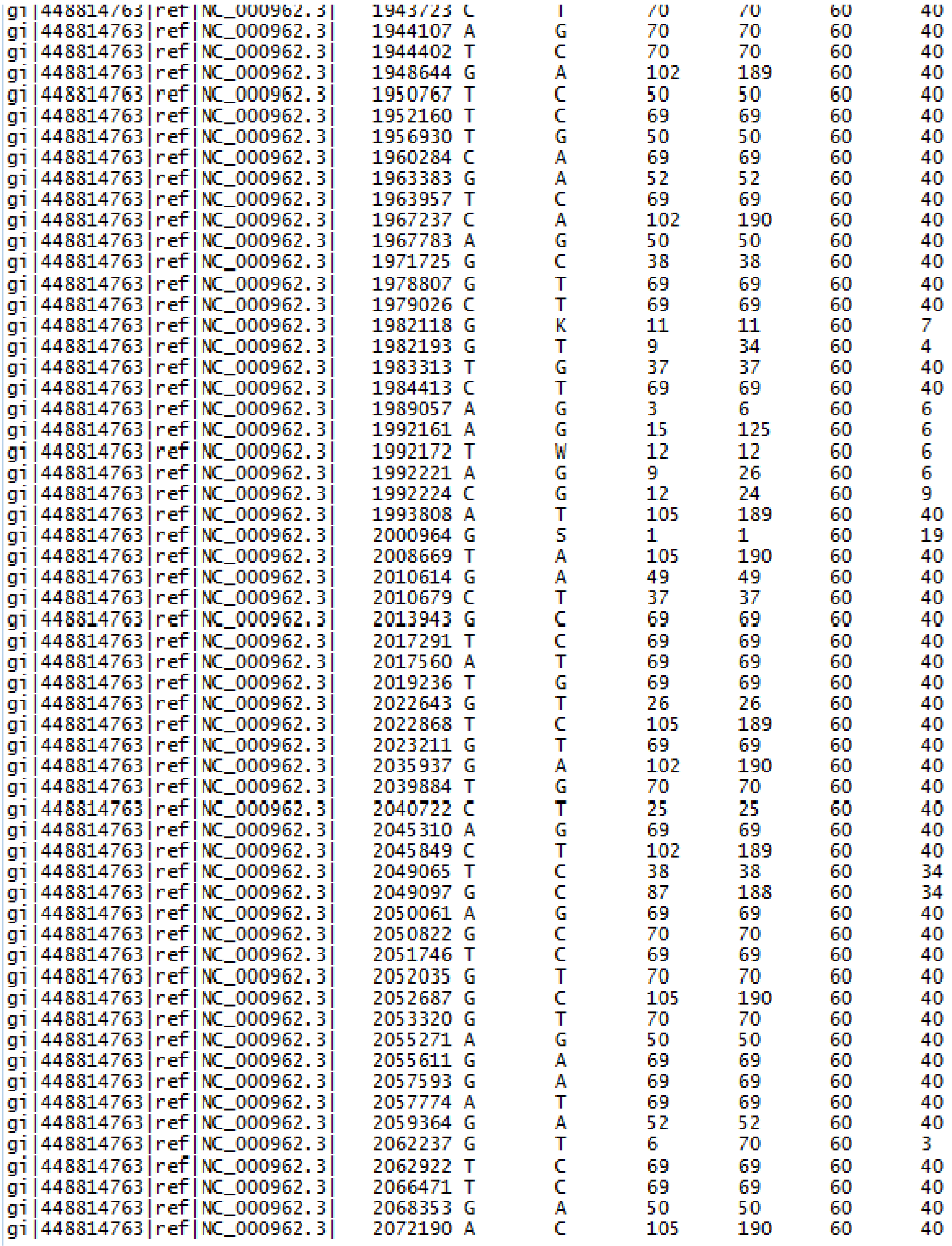

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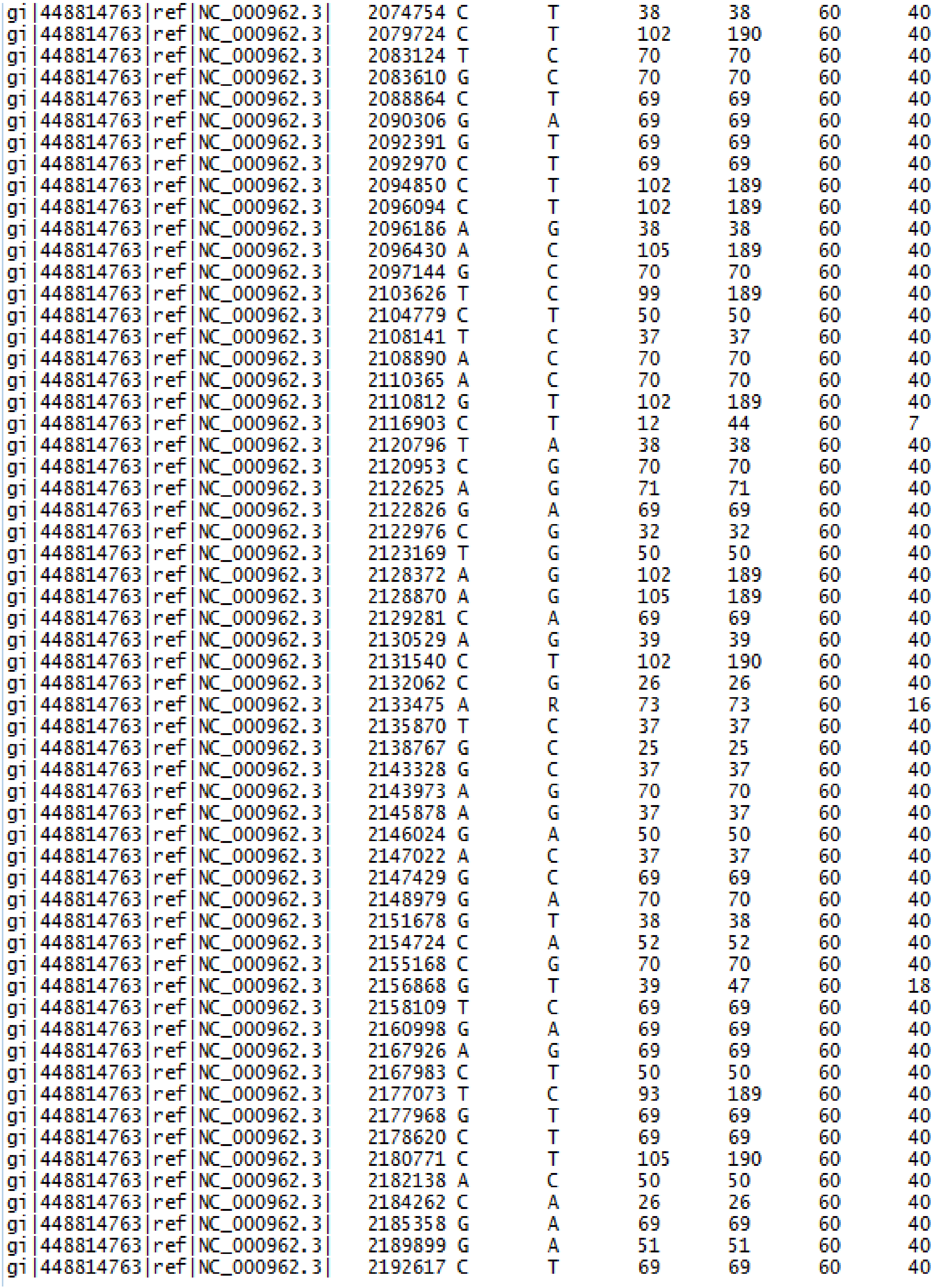

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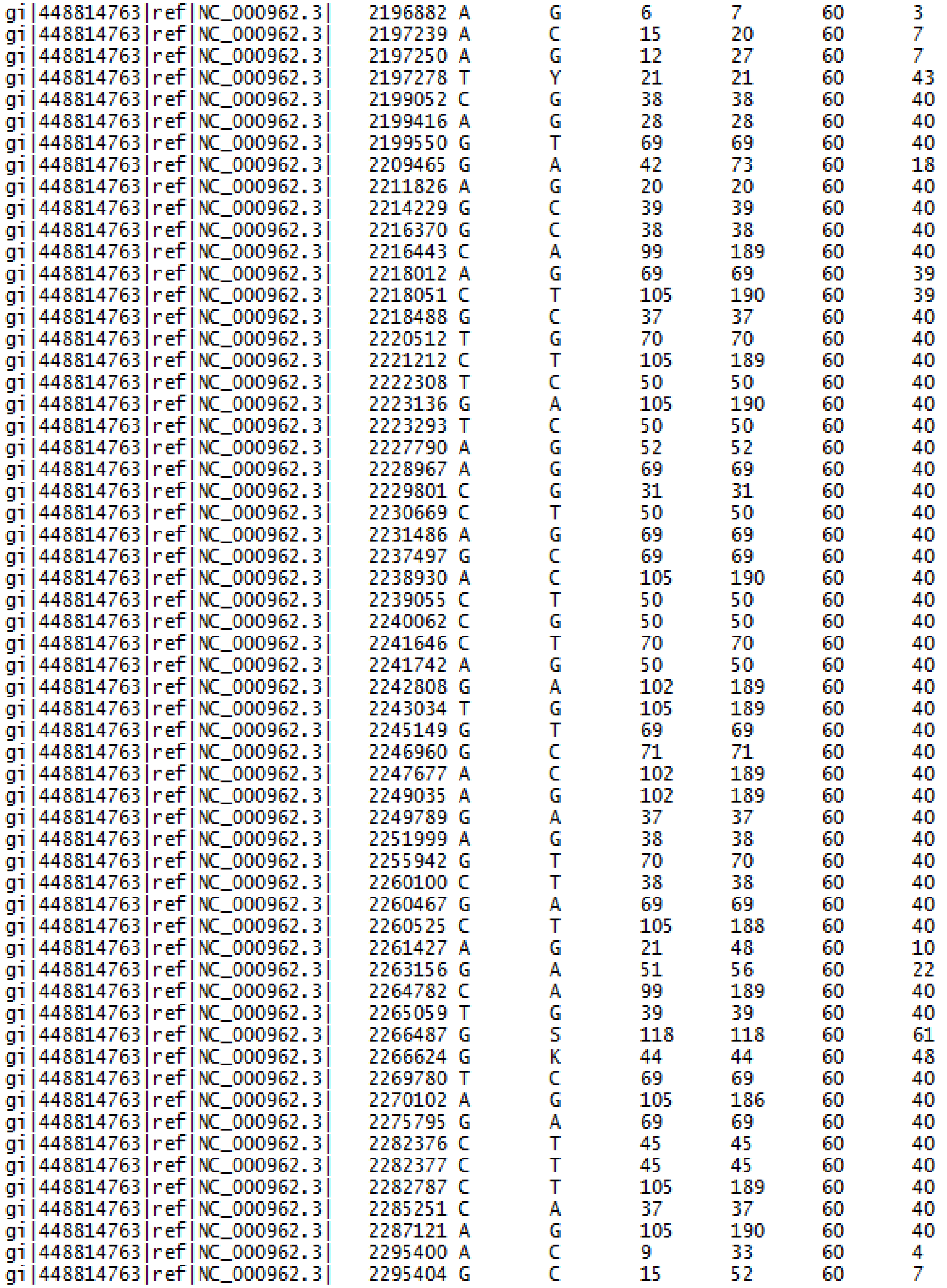

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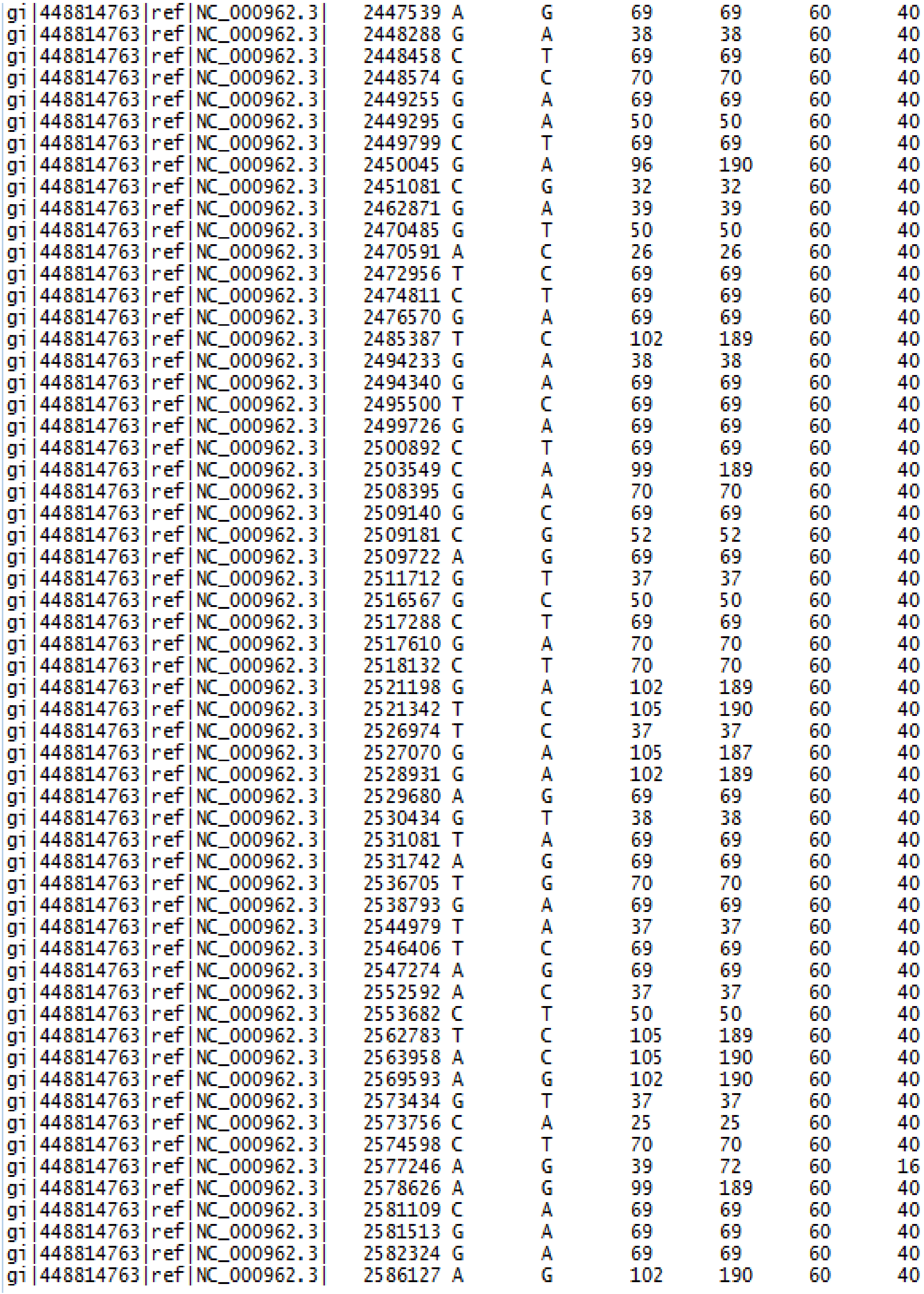

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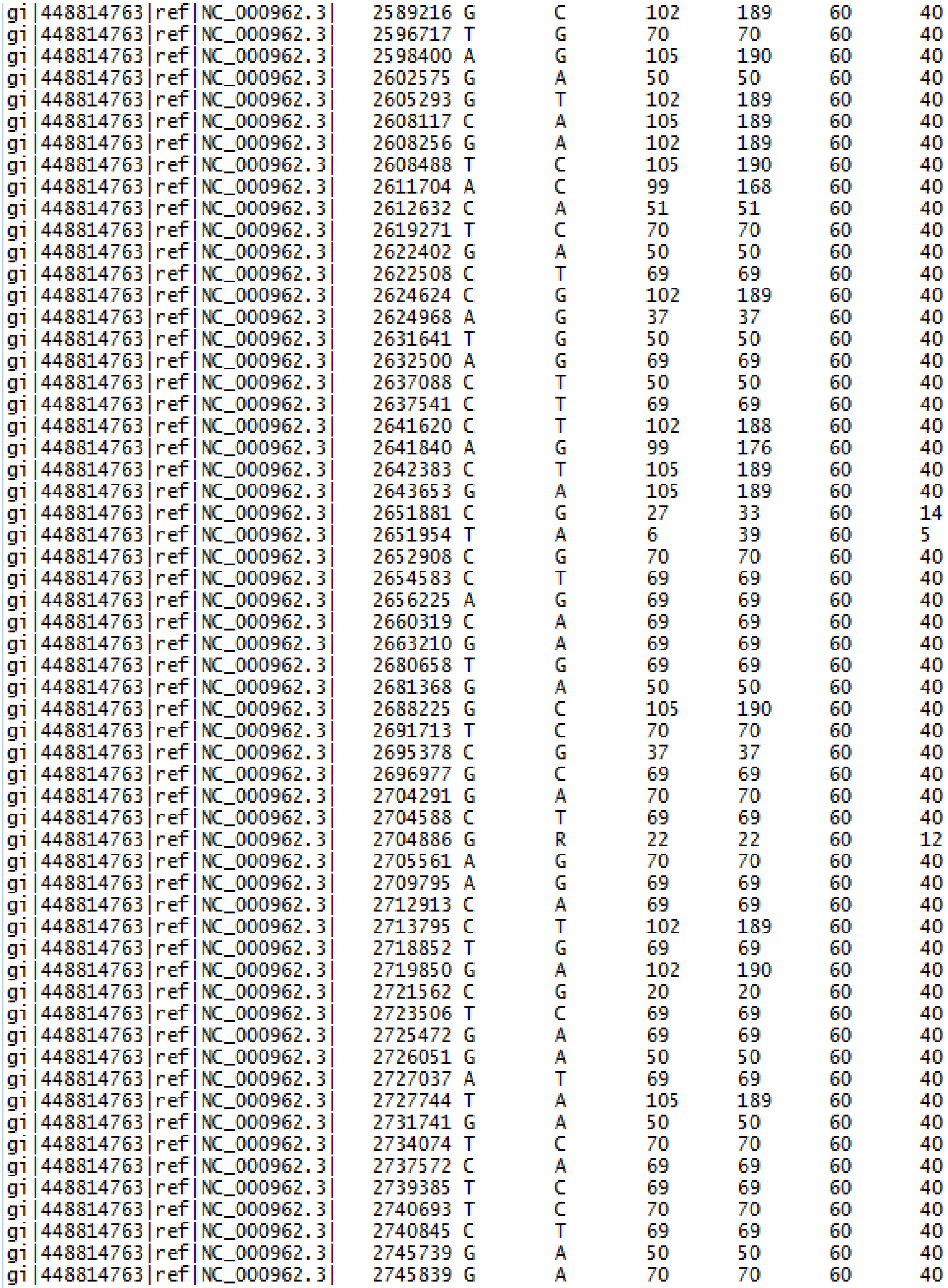

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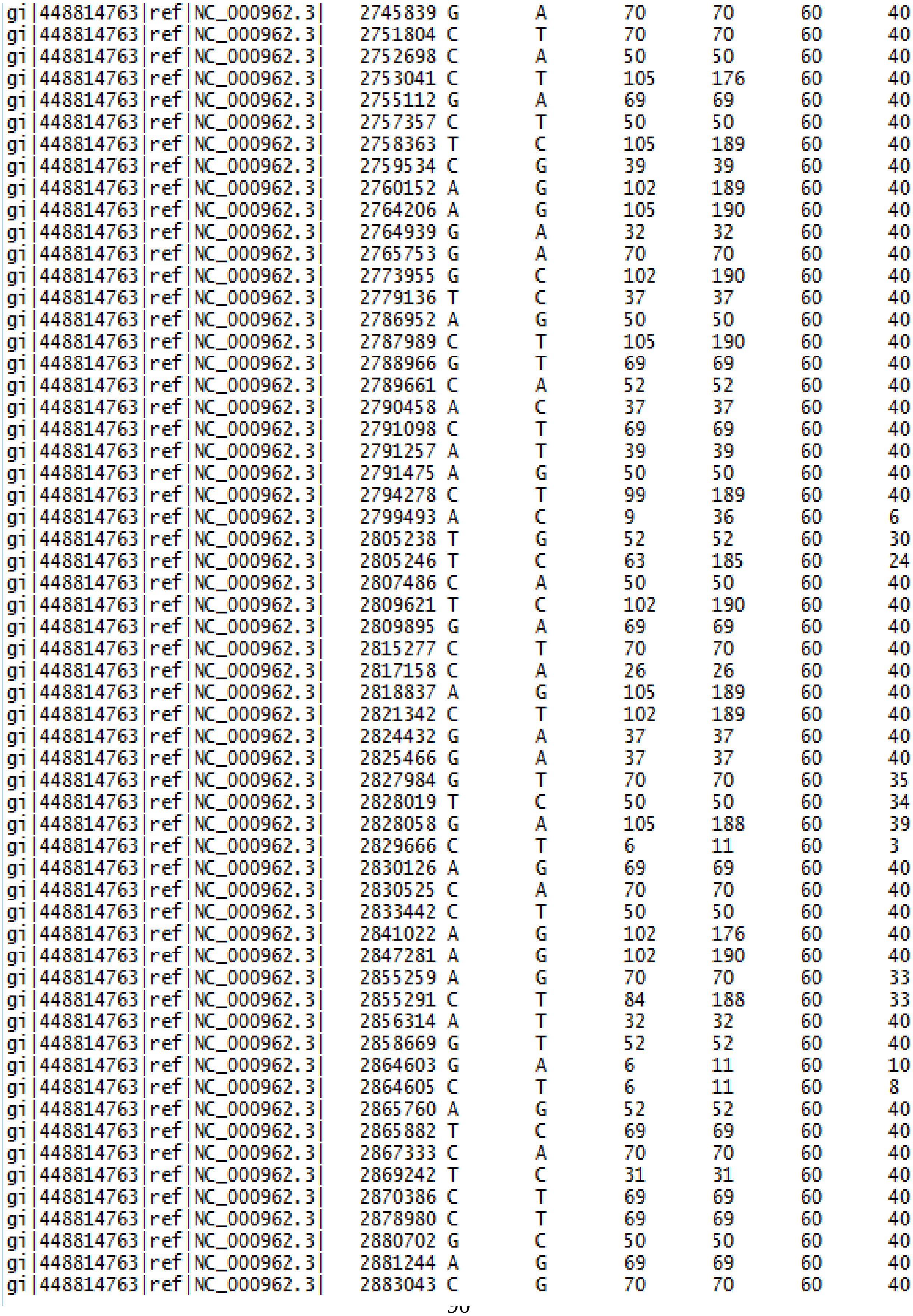

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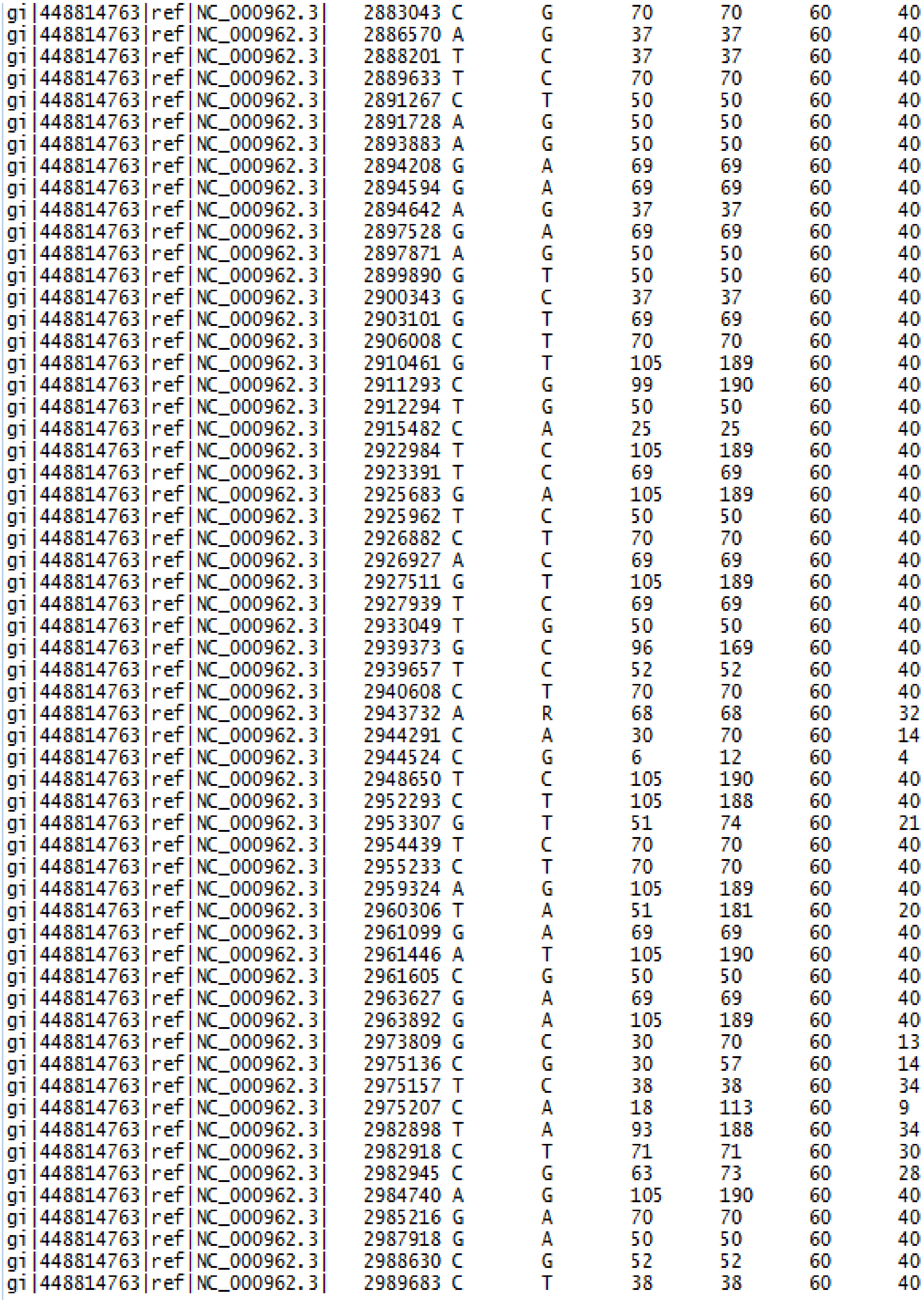

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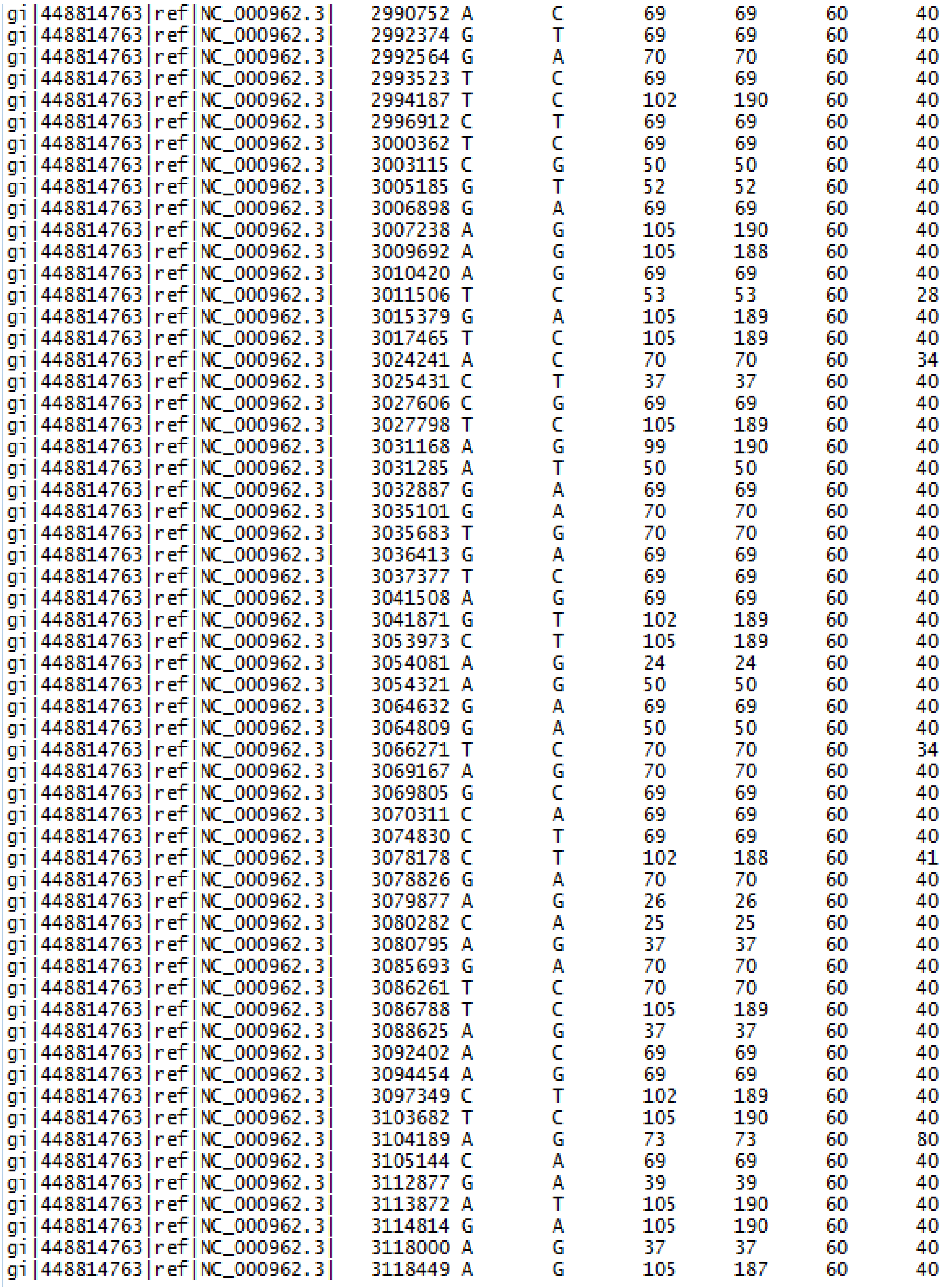

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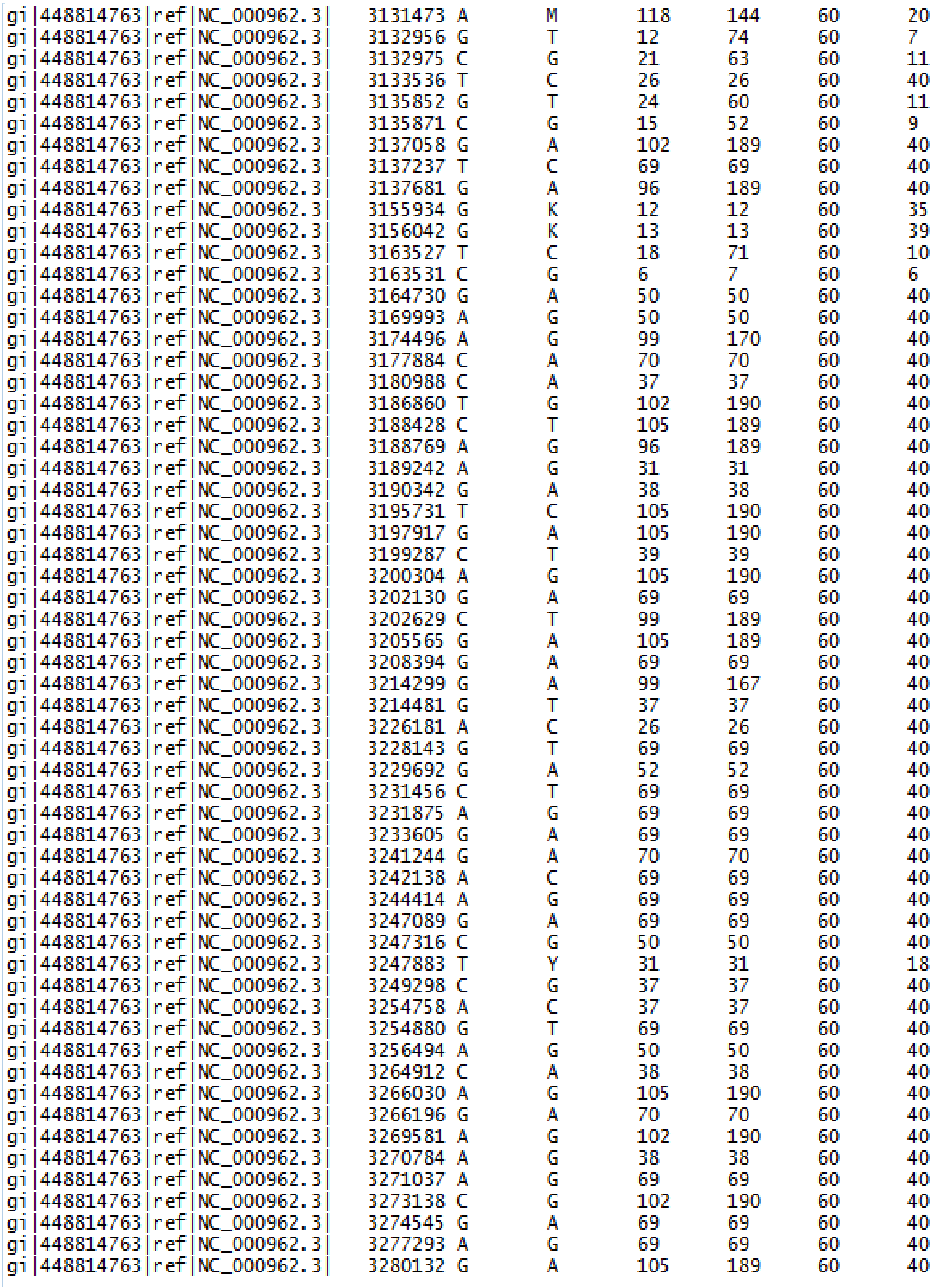

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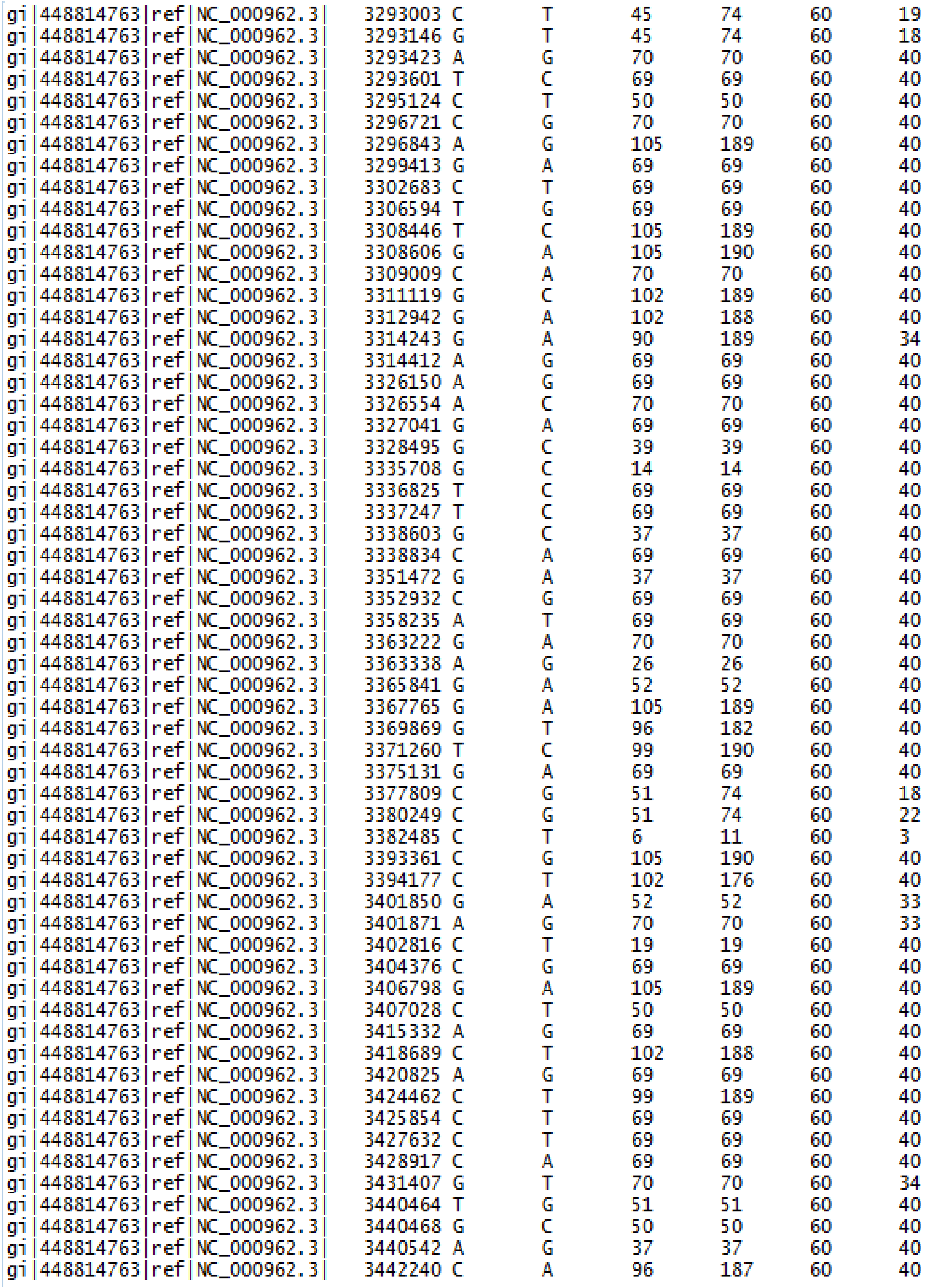

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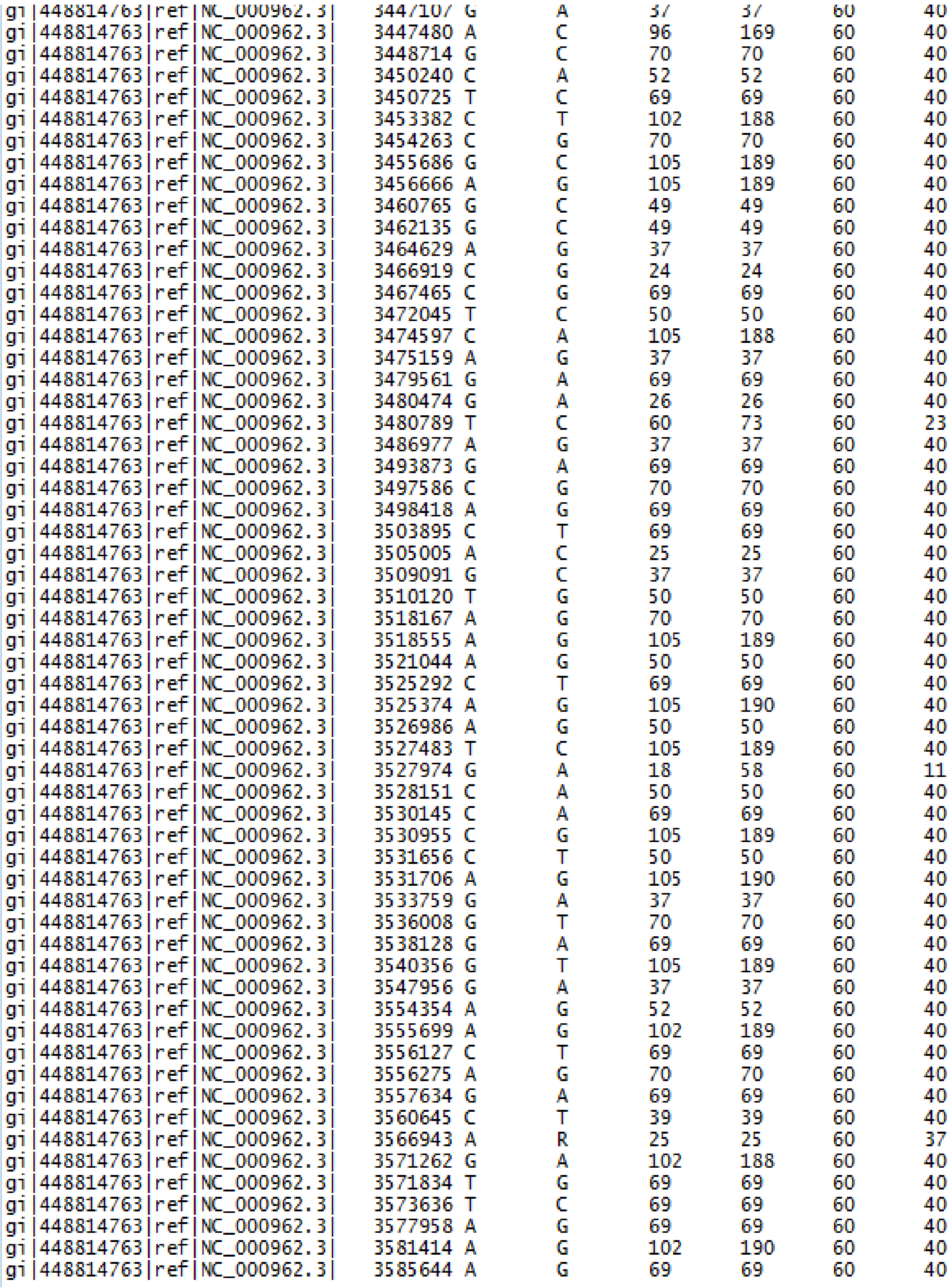

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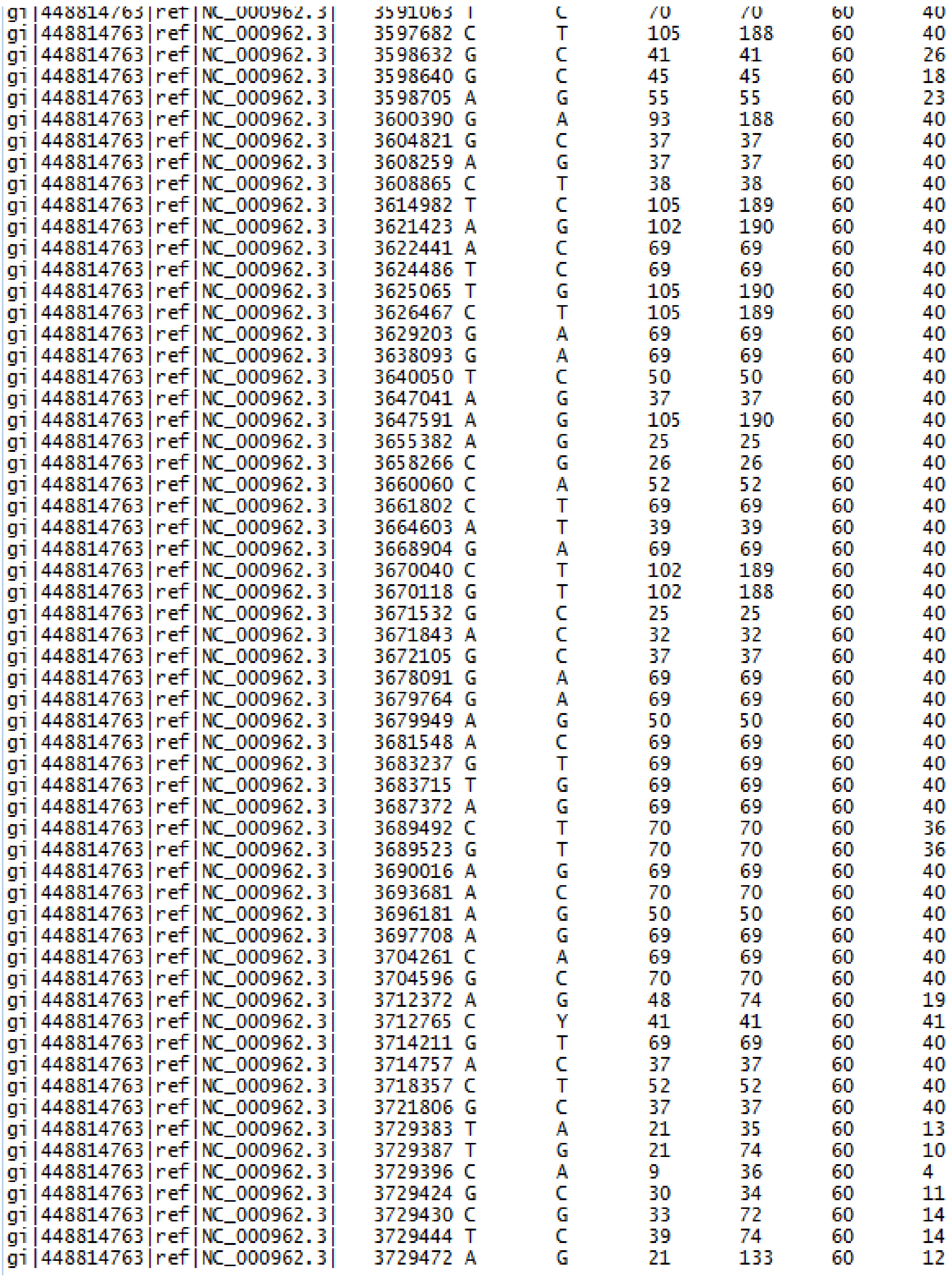

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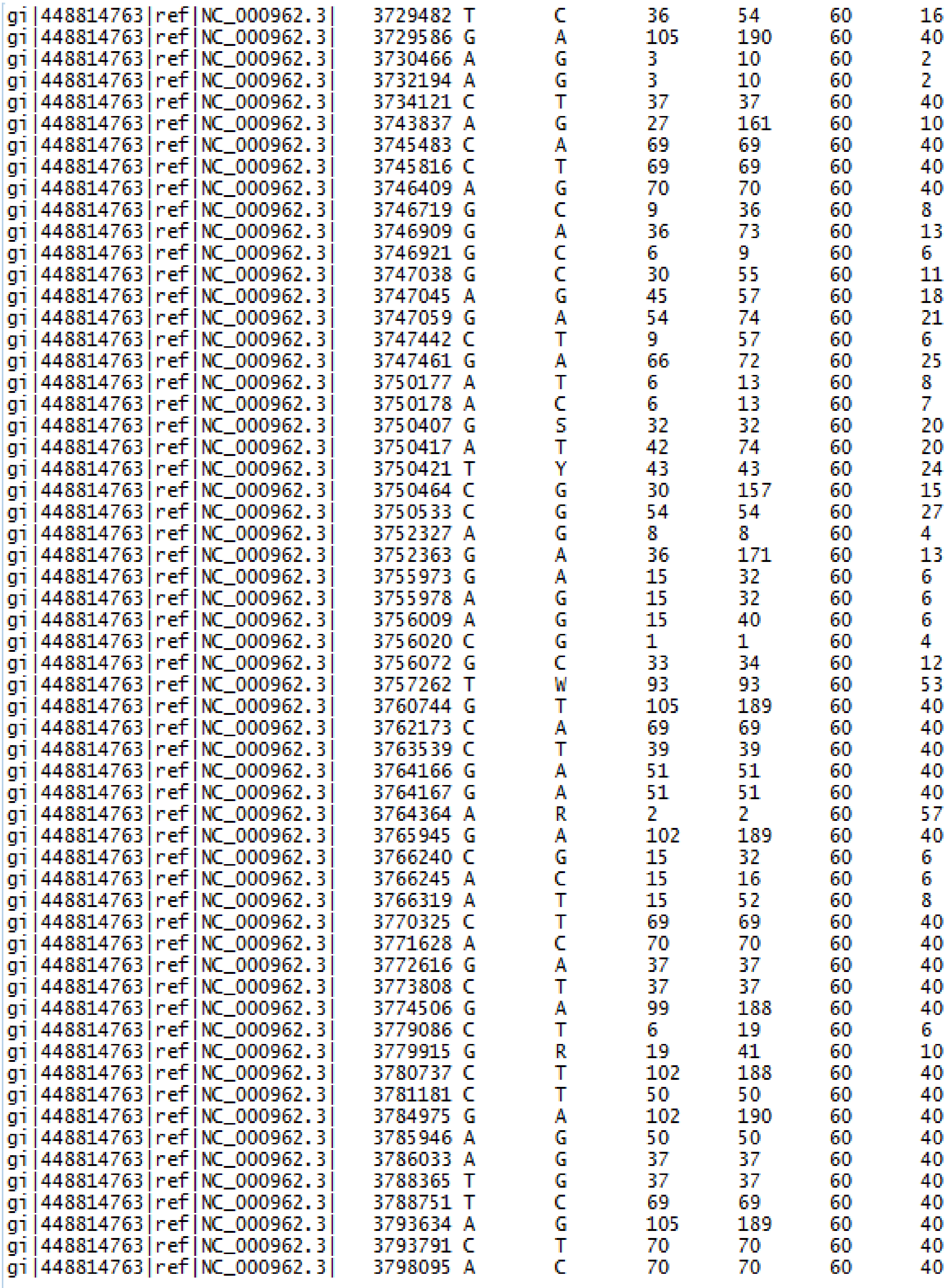

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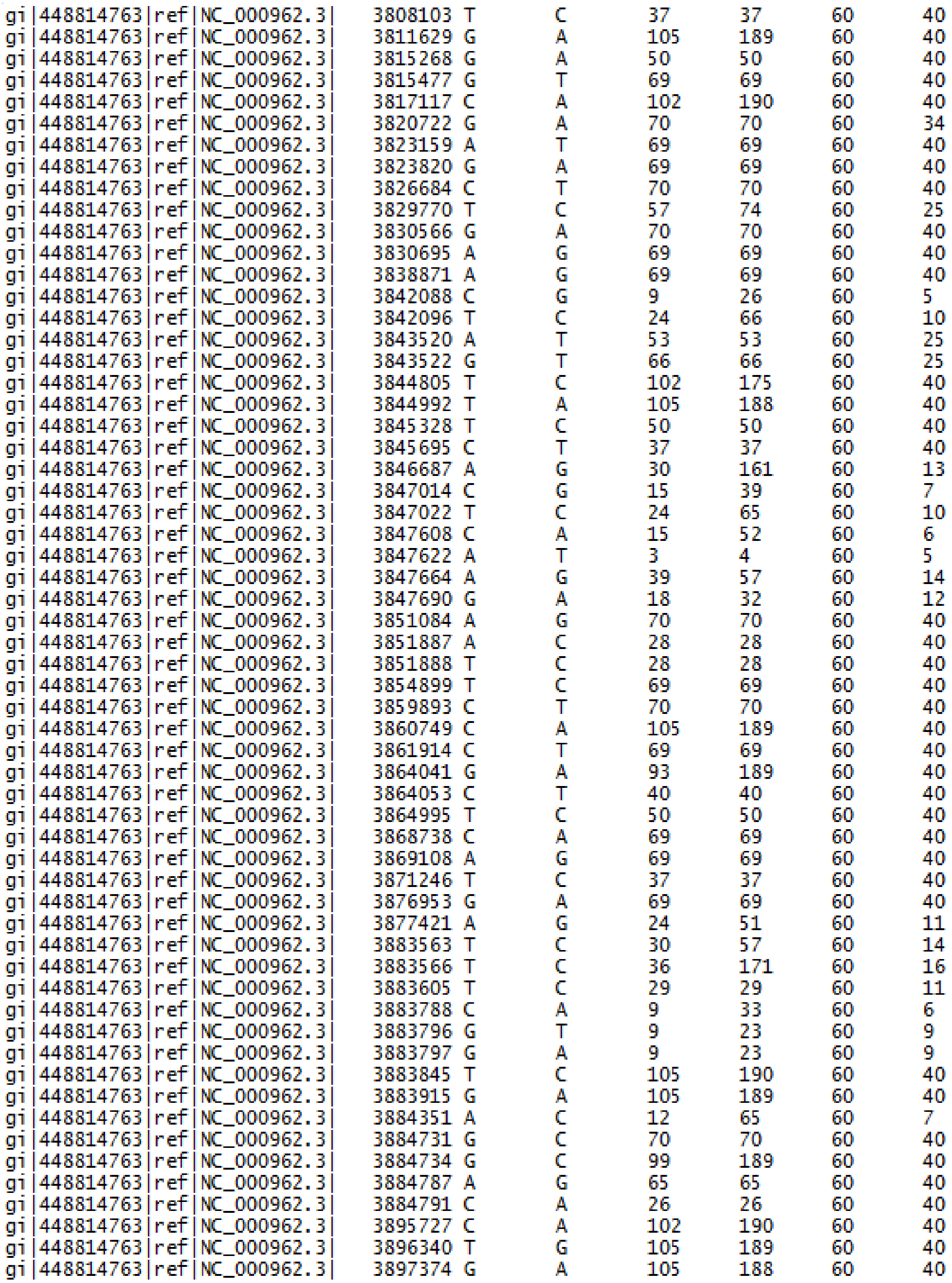

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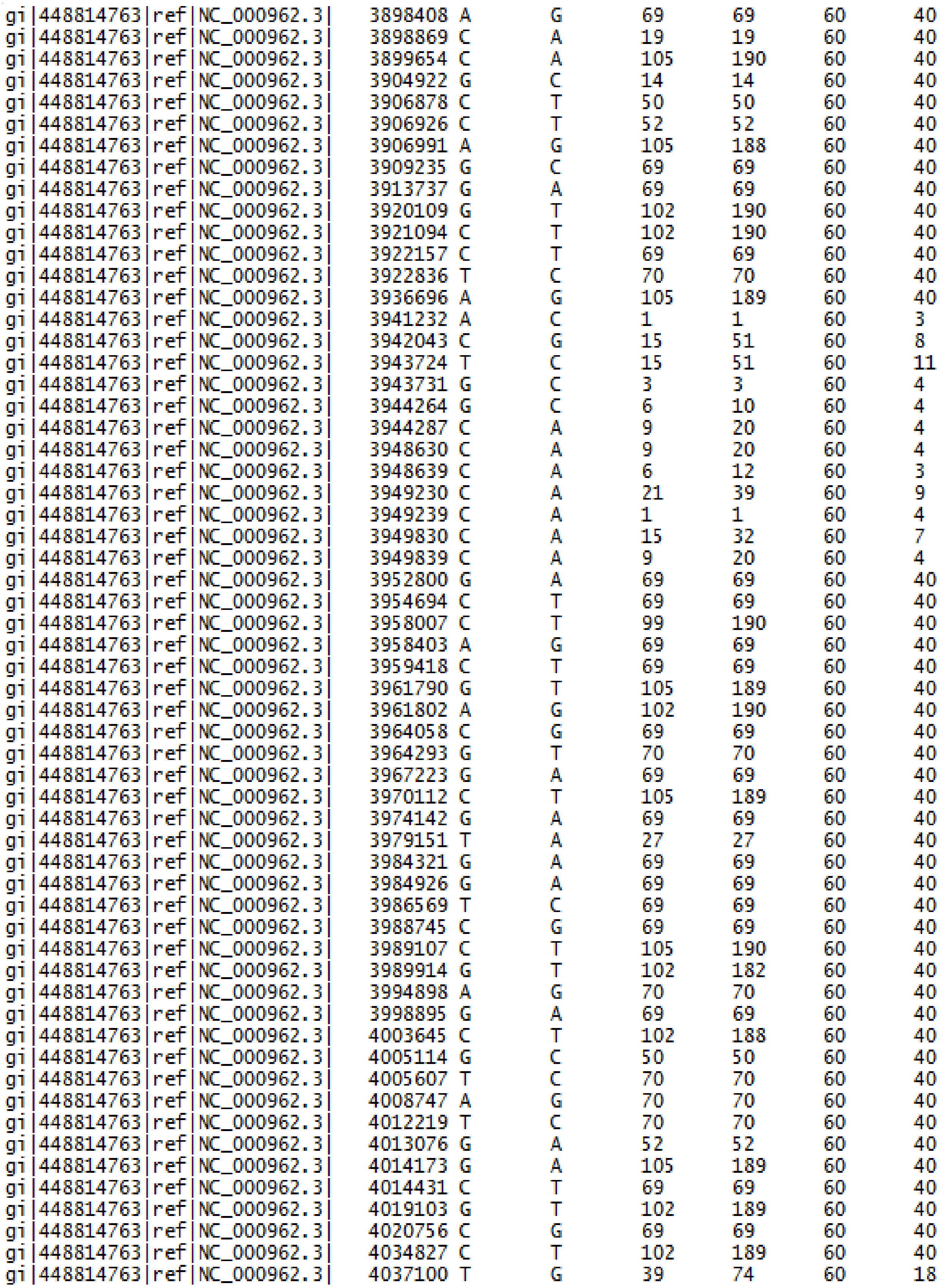

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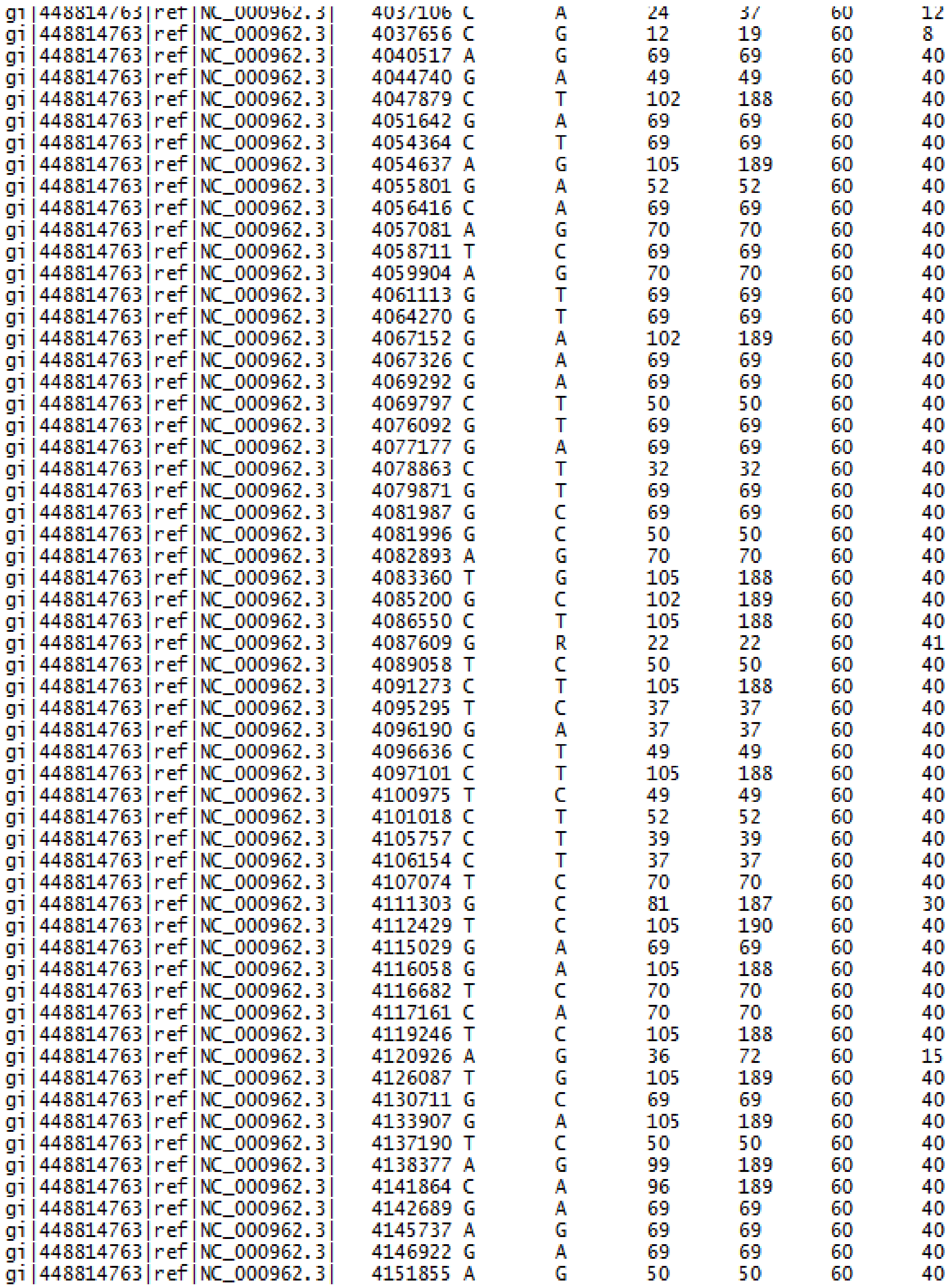

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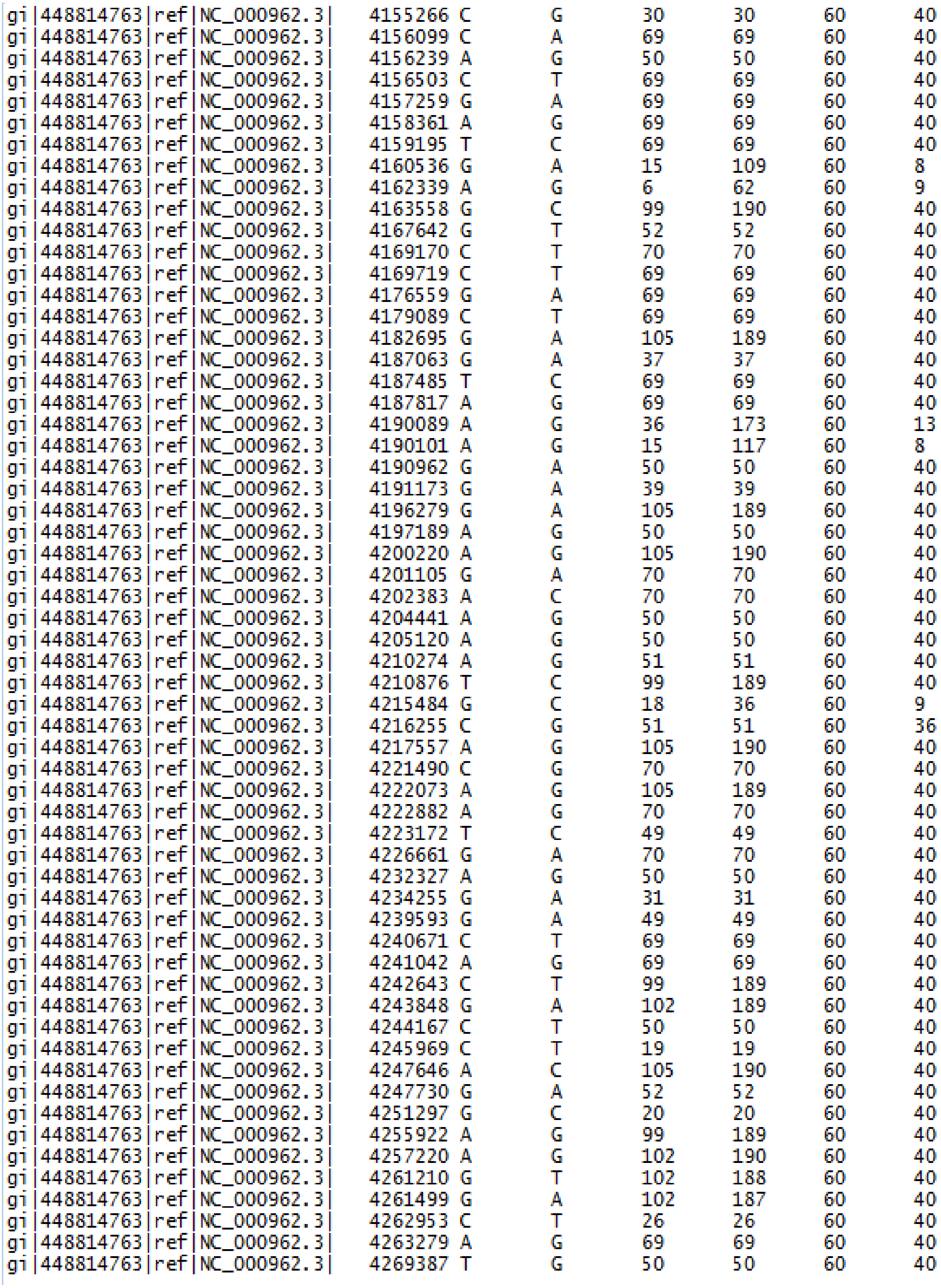

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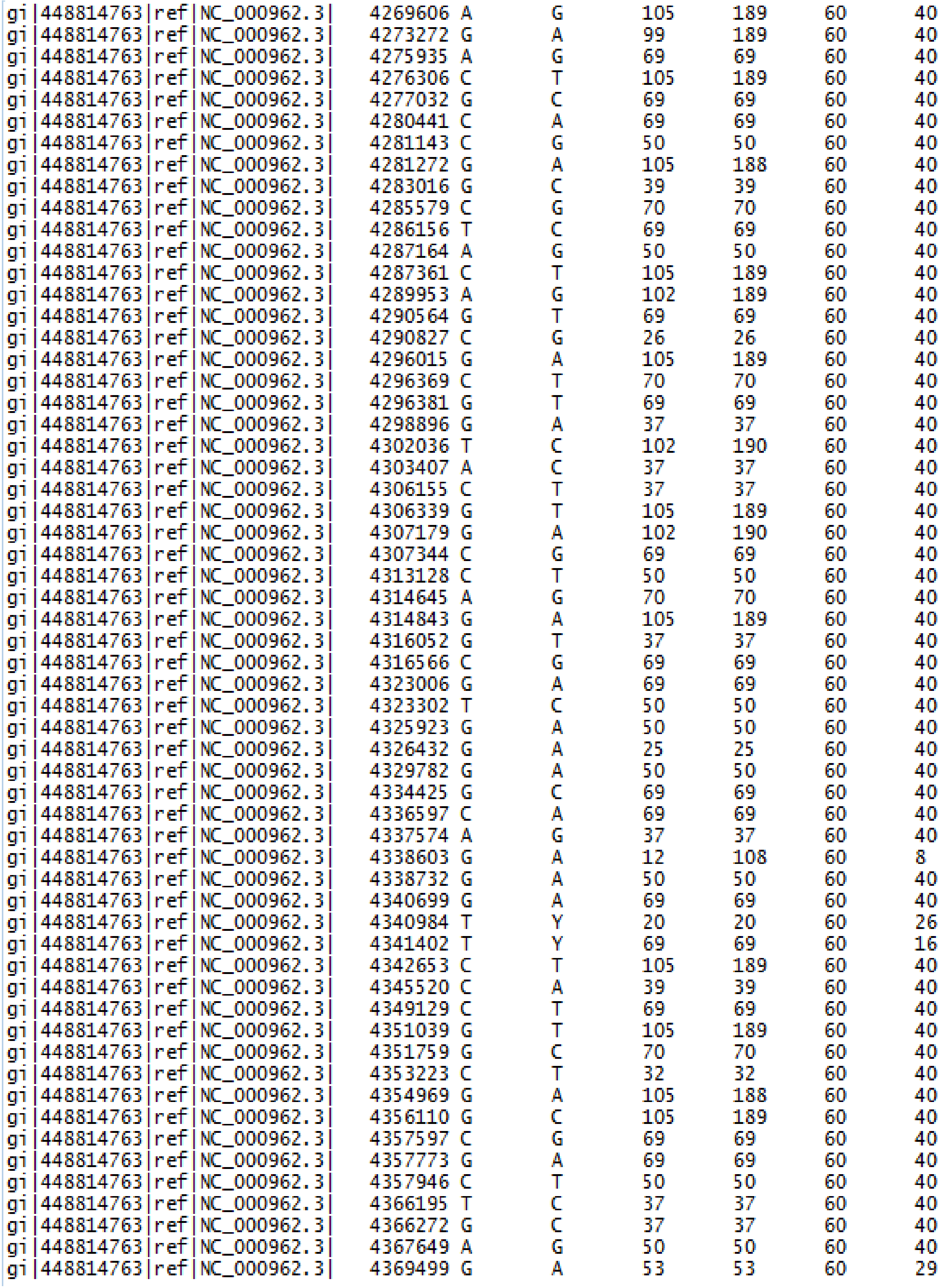

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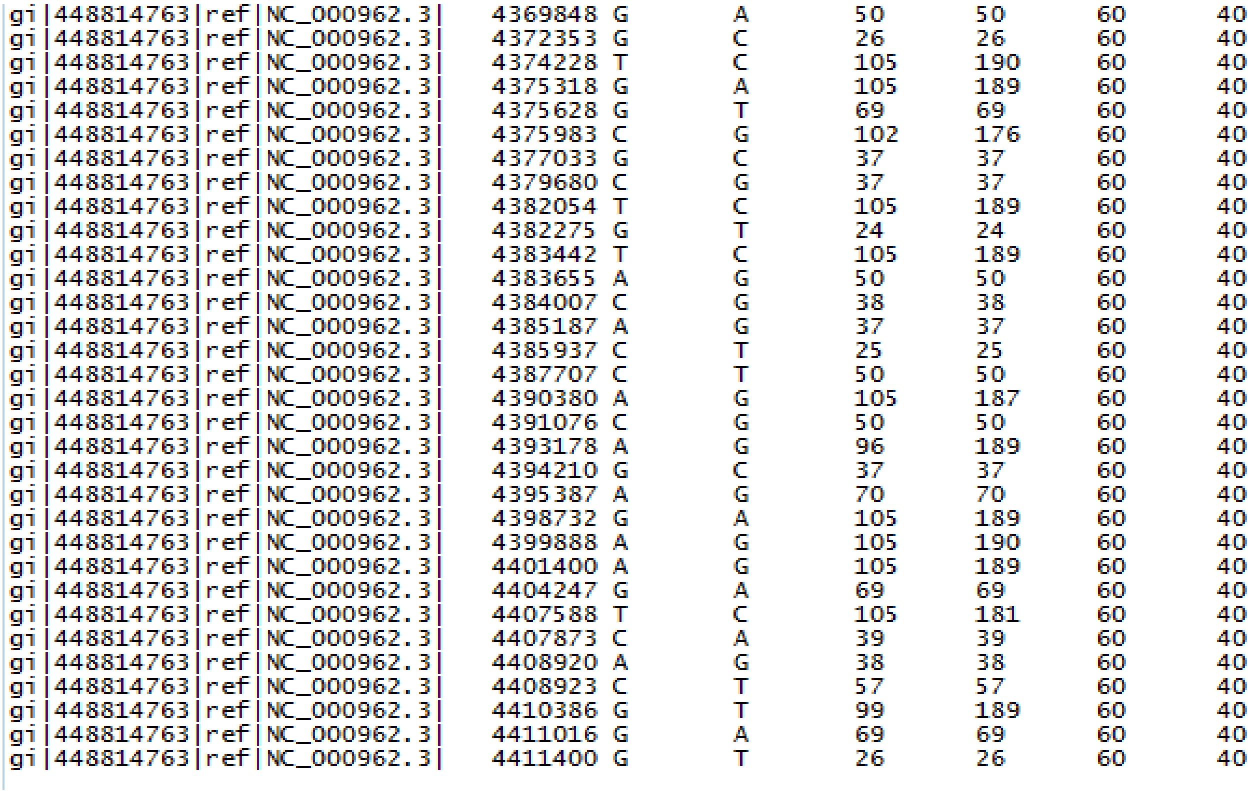

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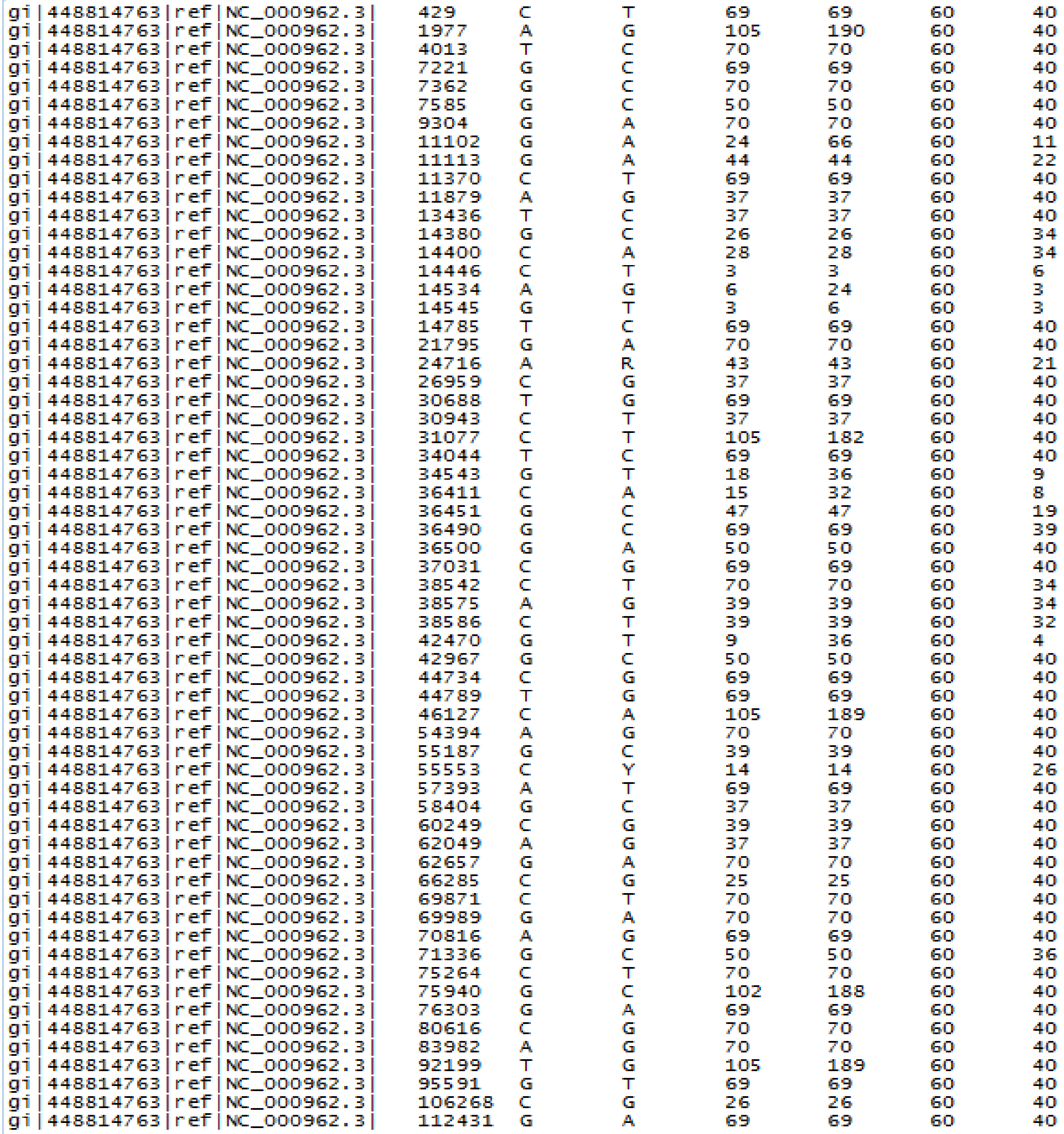
APPENDIX III :-

Result of alignment of AMXW strain with *Mycobacterium tuberculosis* H37Rv

Chromosome Coordinate ref. Consensus Consensus SNP MAPQ Depth
Base Base Quality Quality



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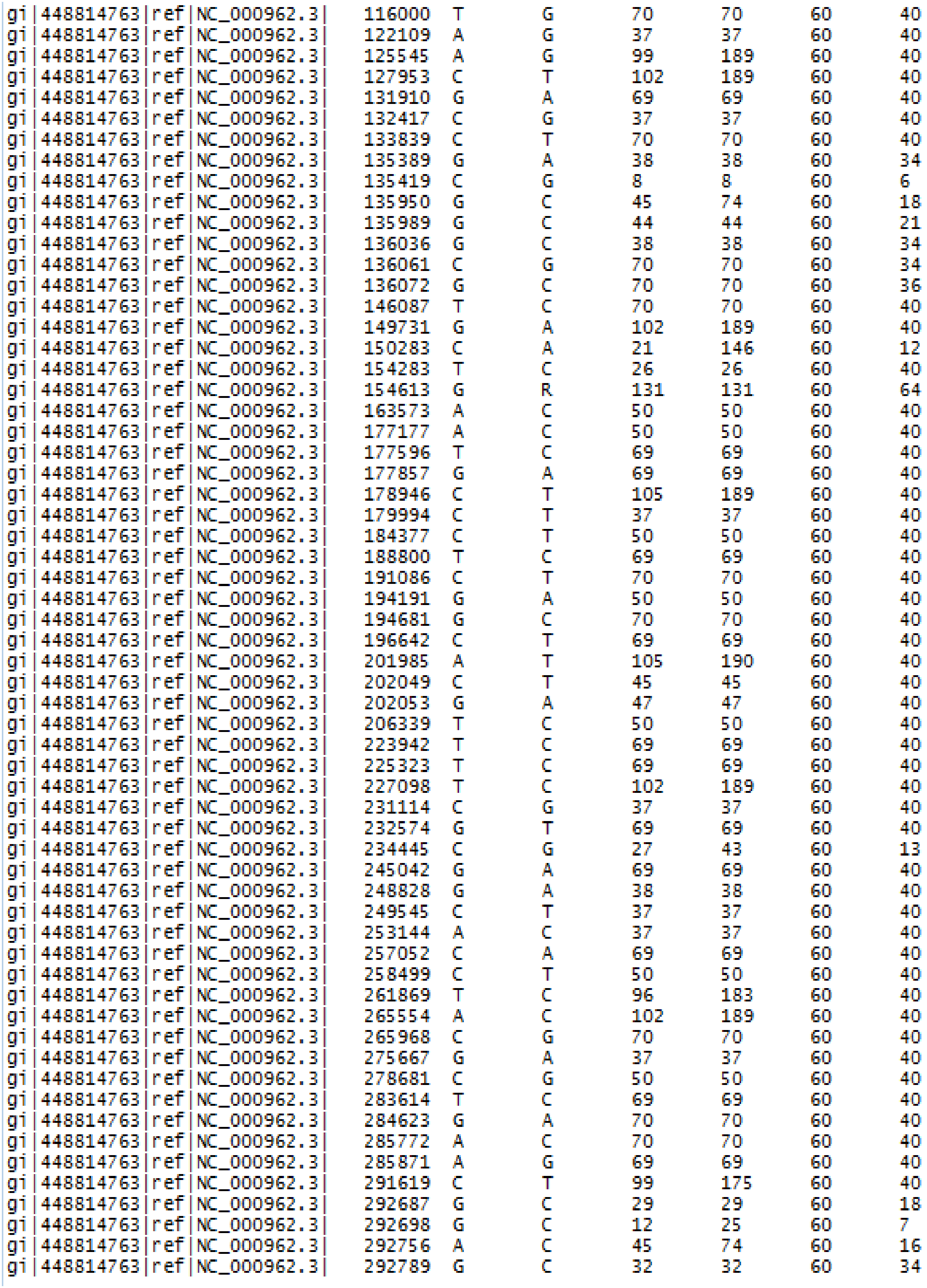

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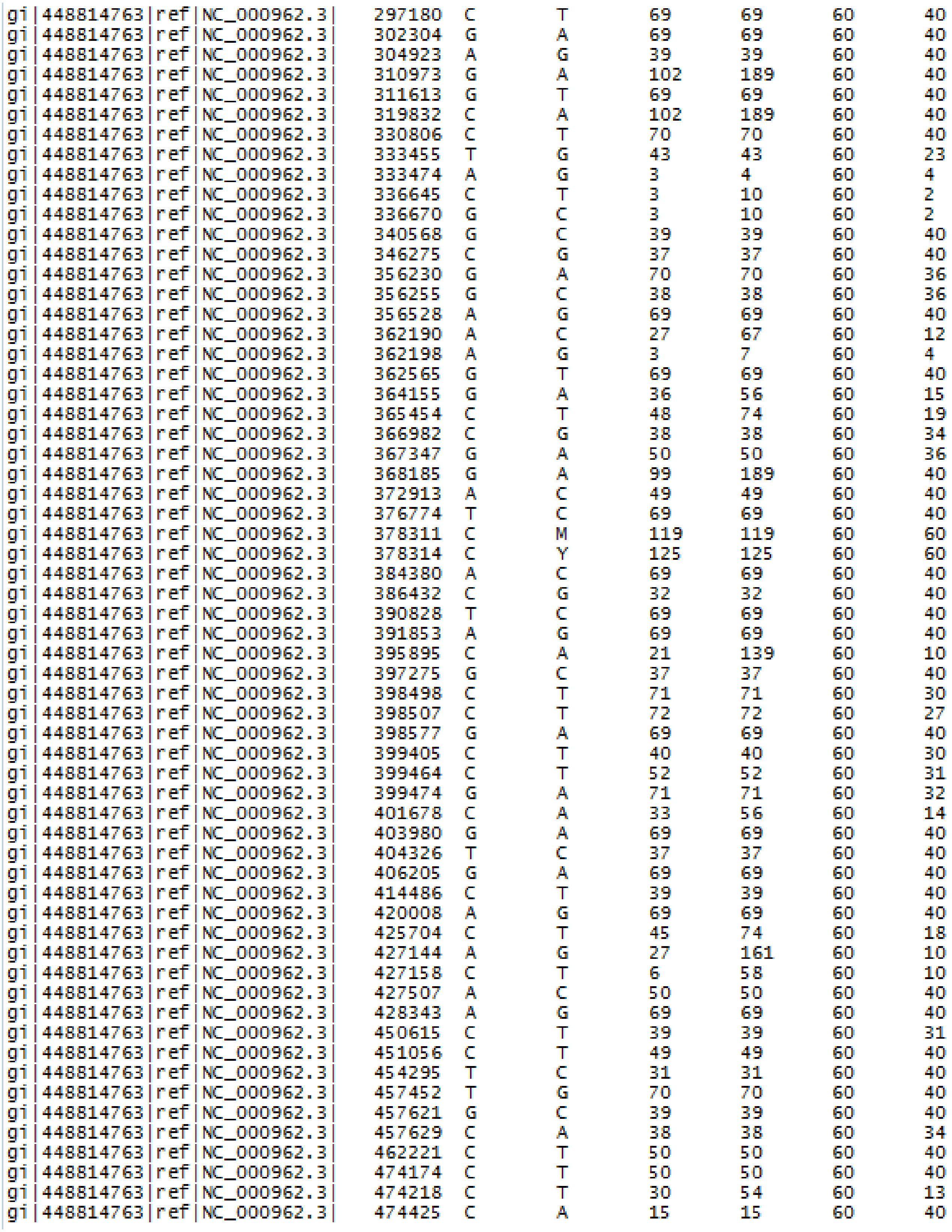

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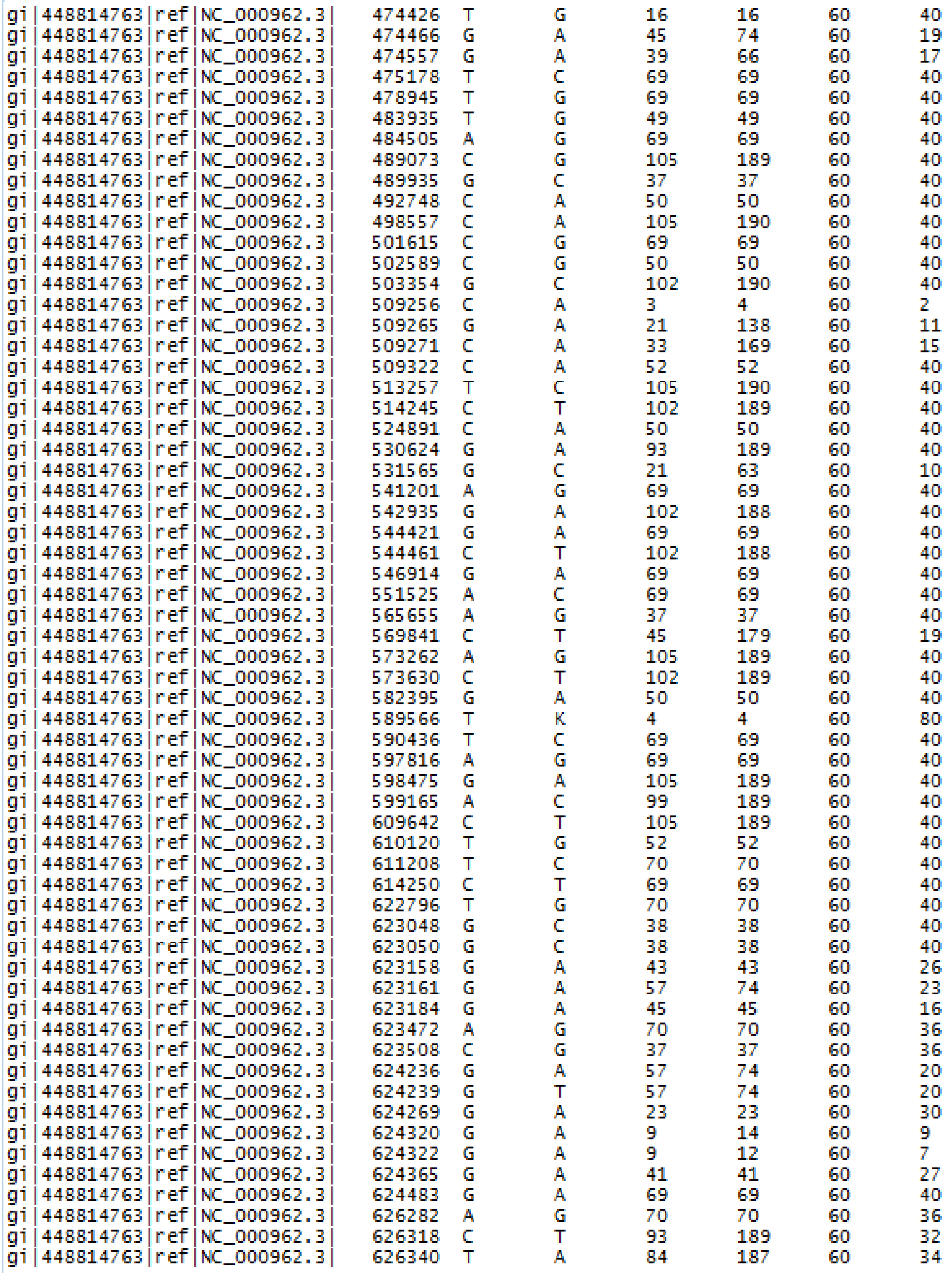

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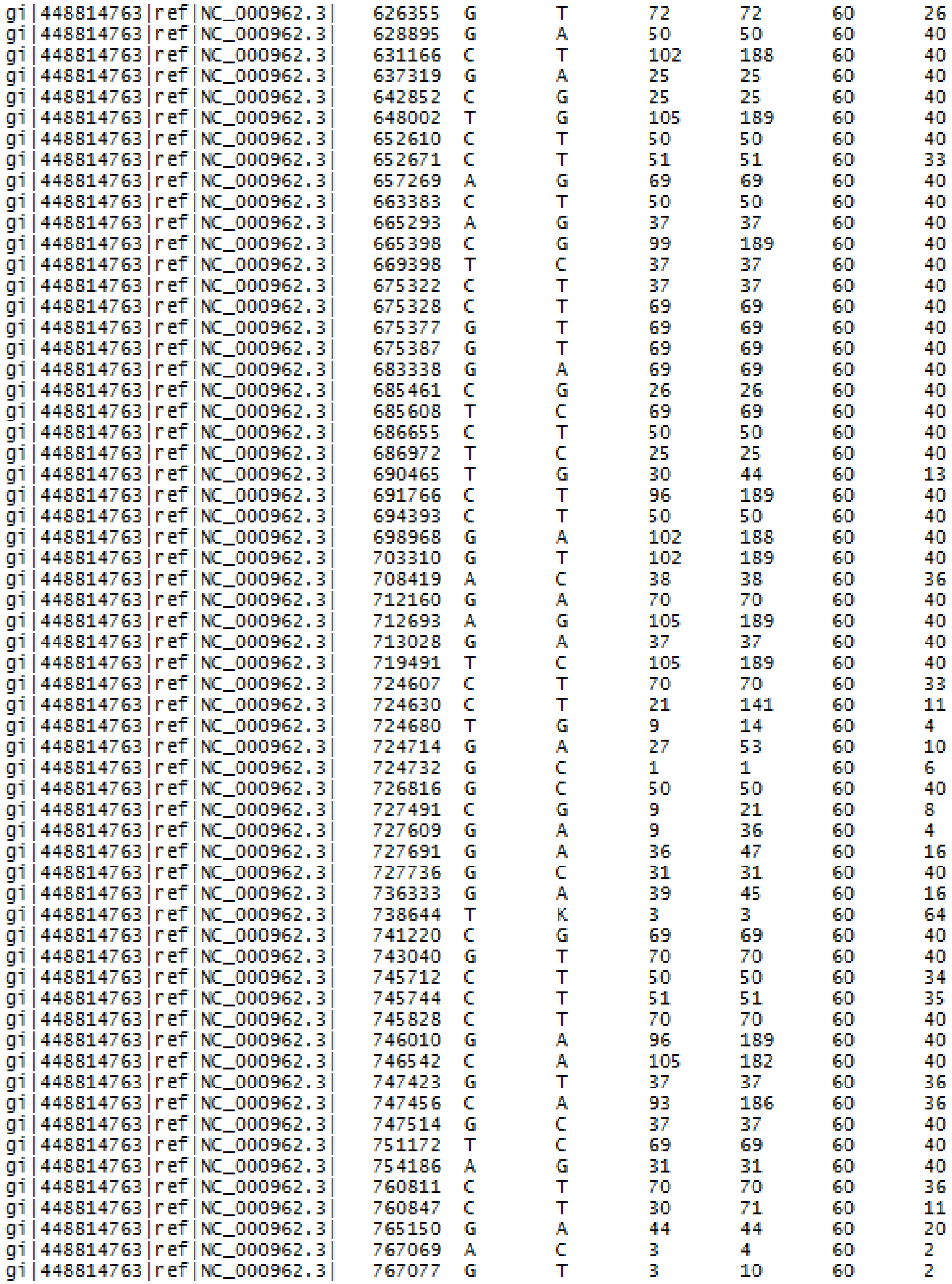

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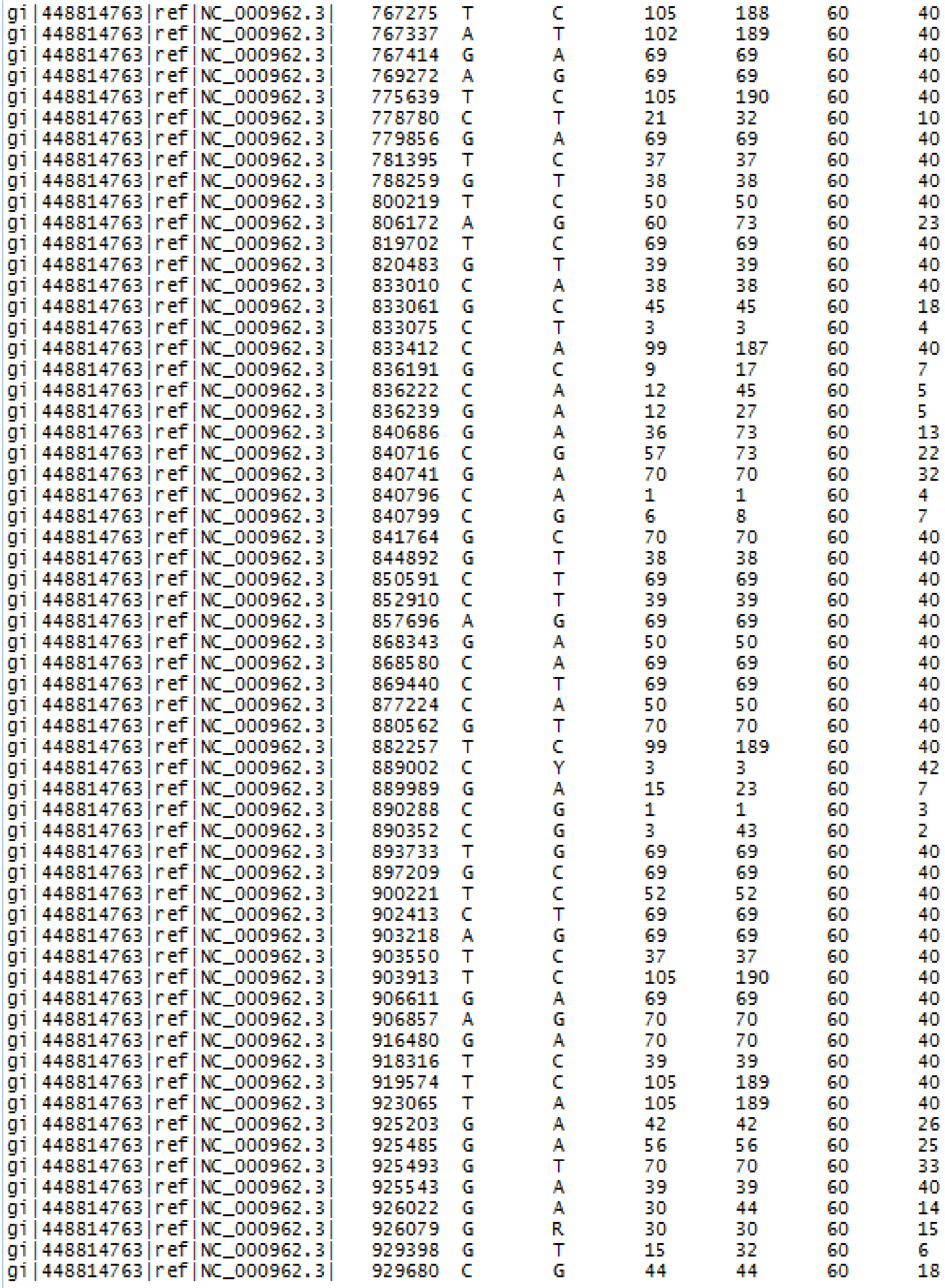

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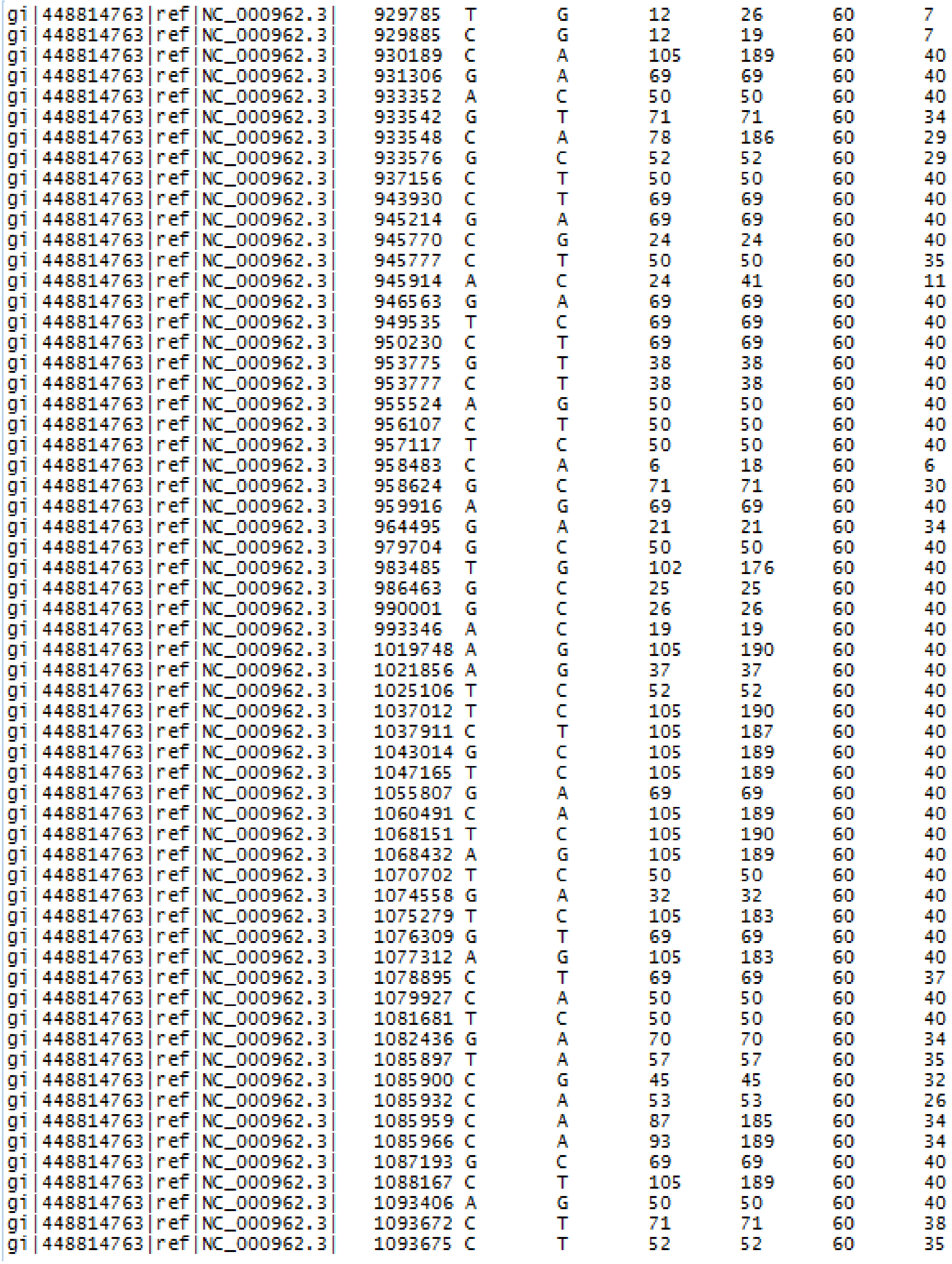

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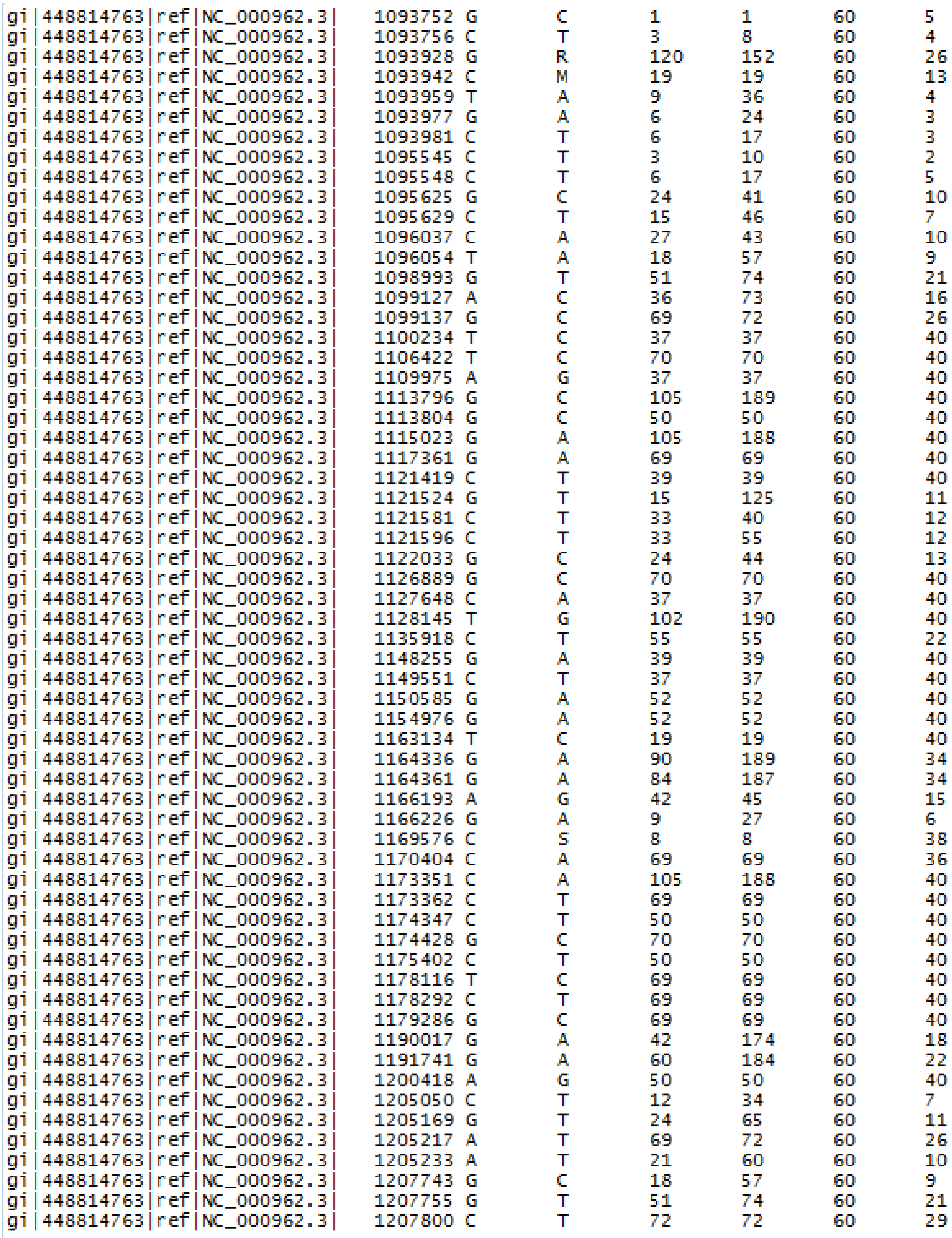

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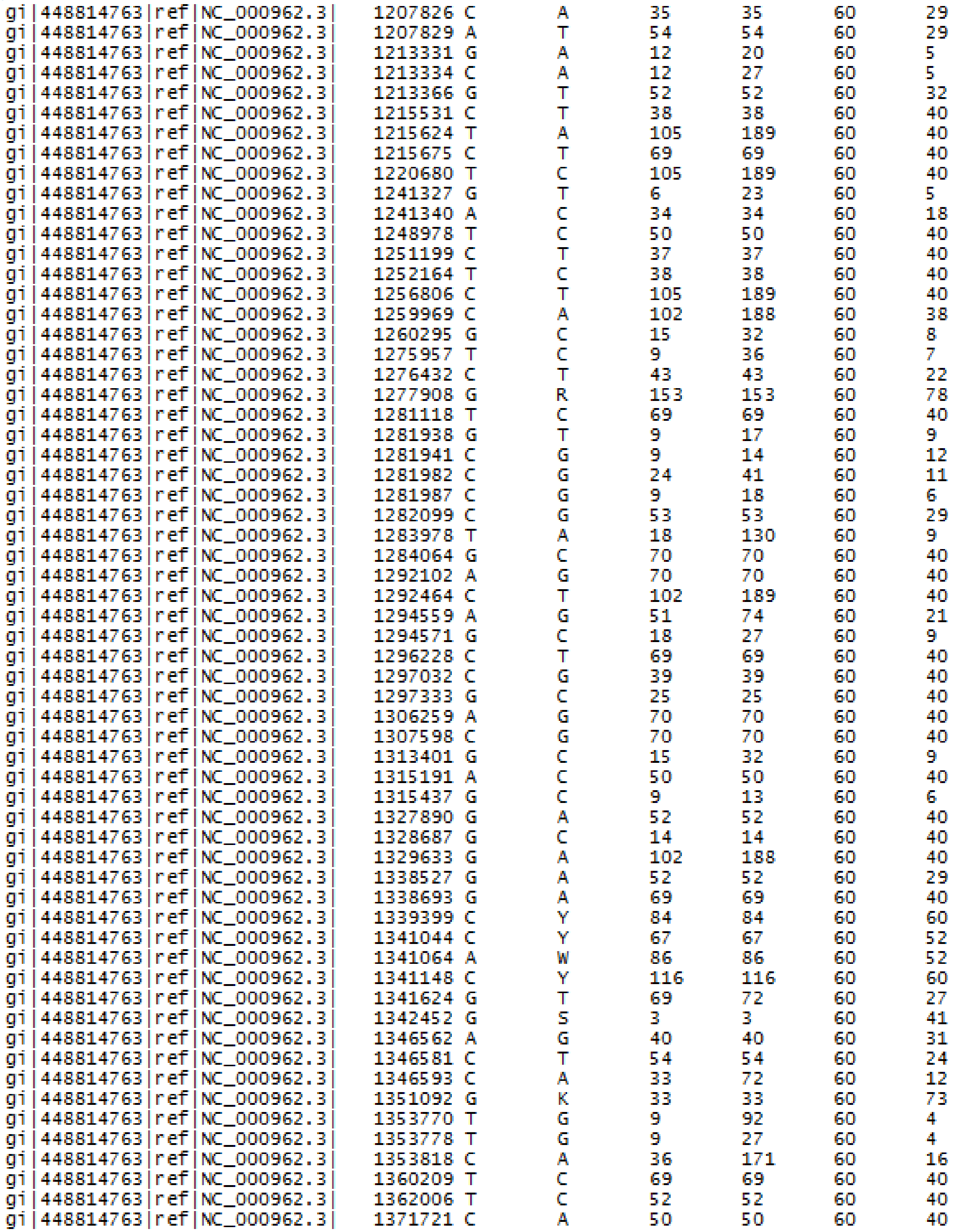

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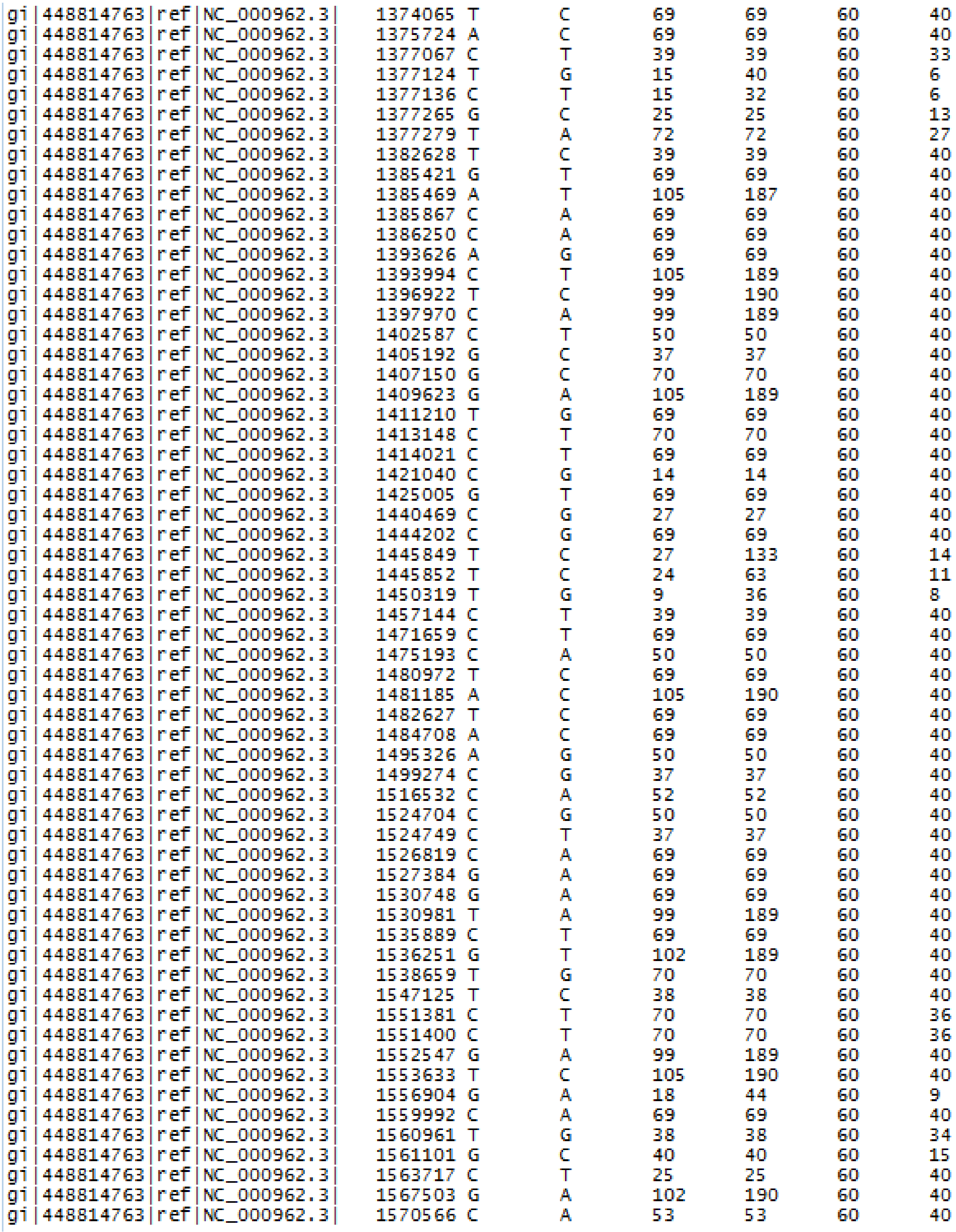

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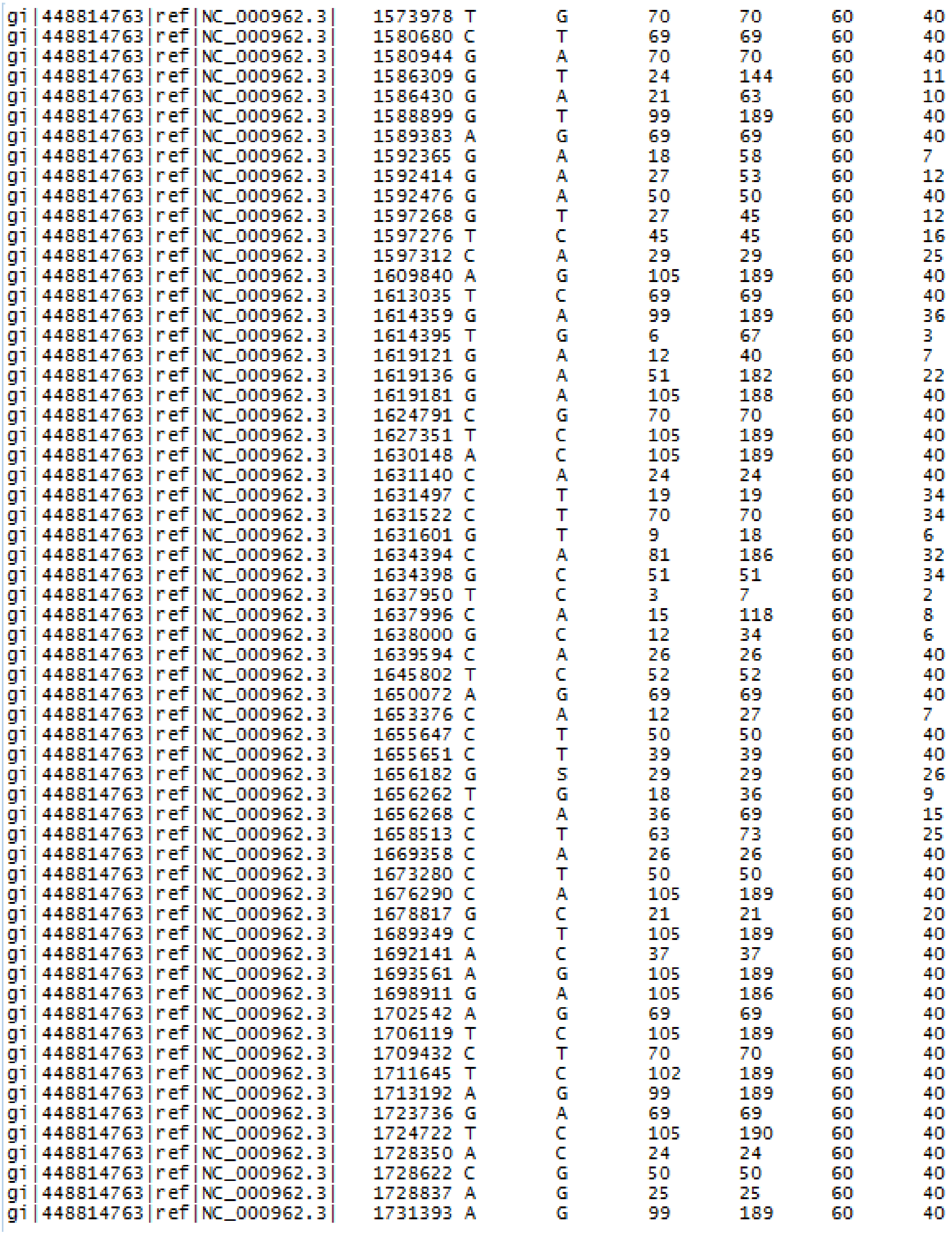

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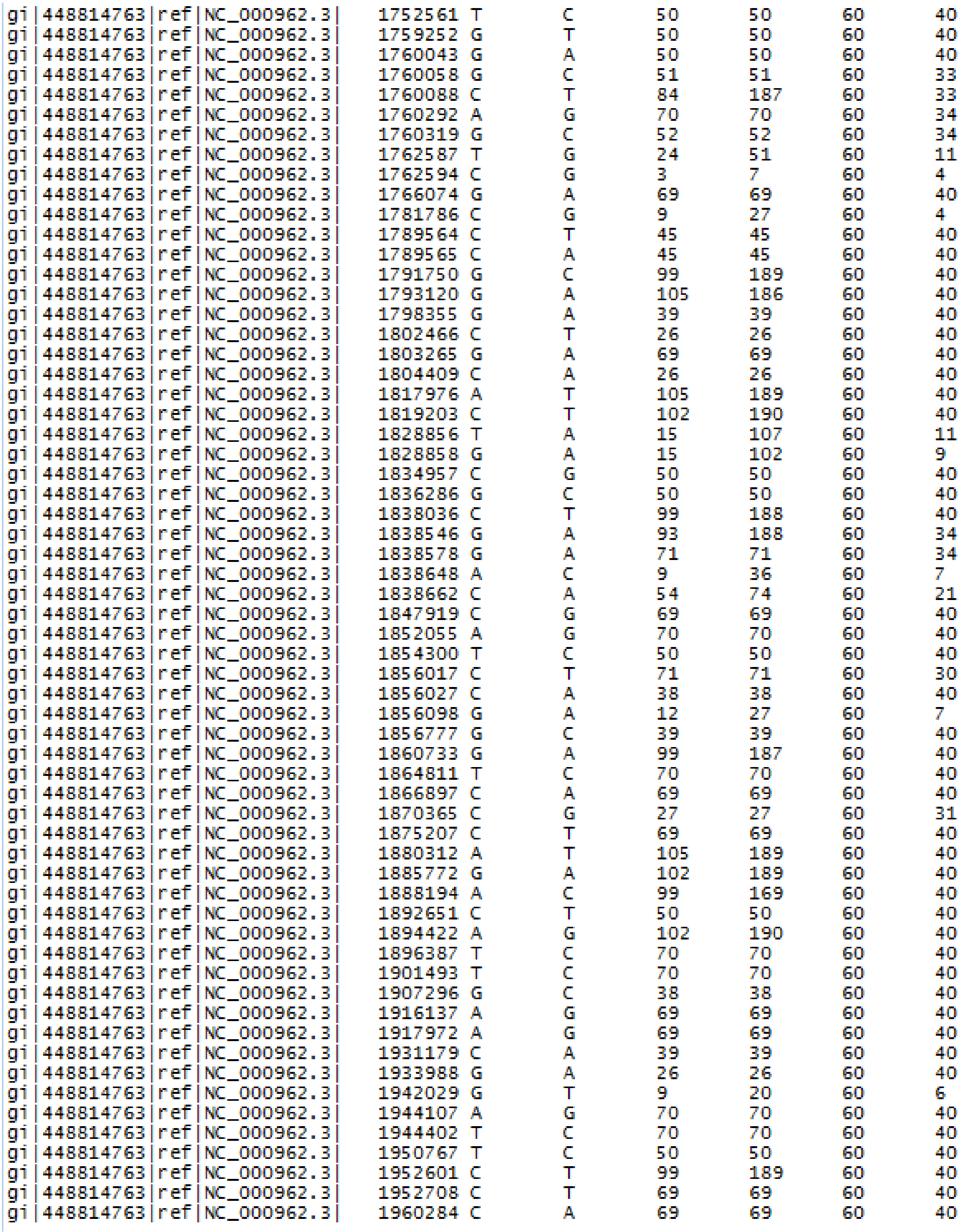

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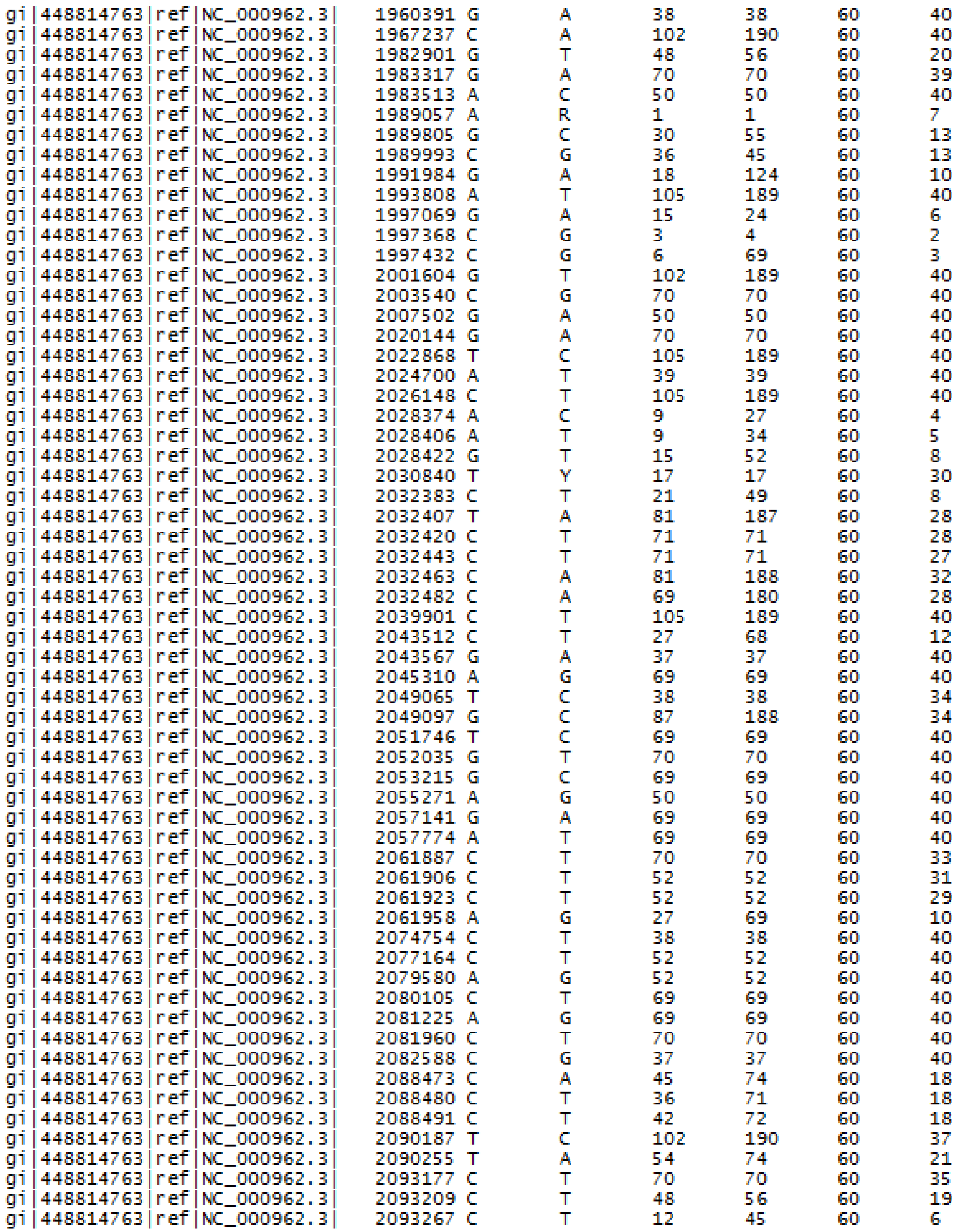

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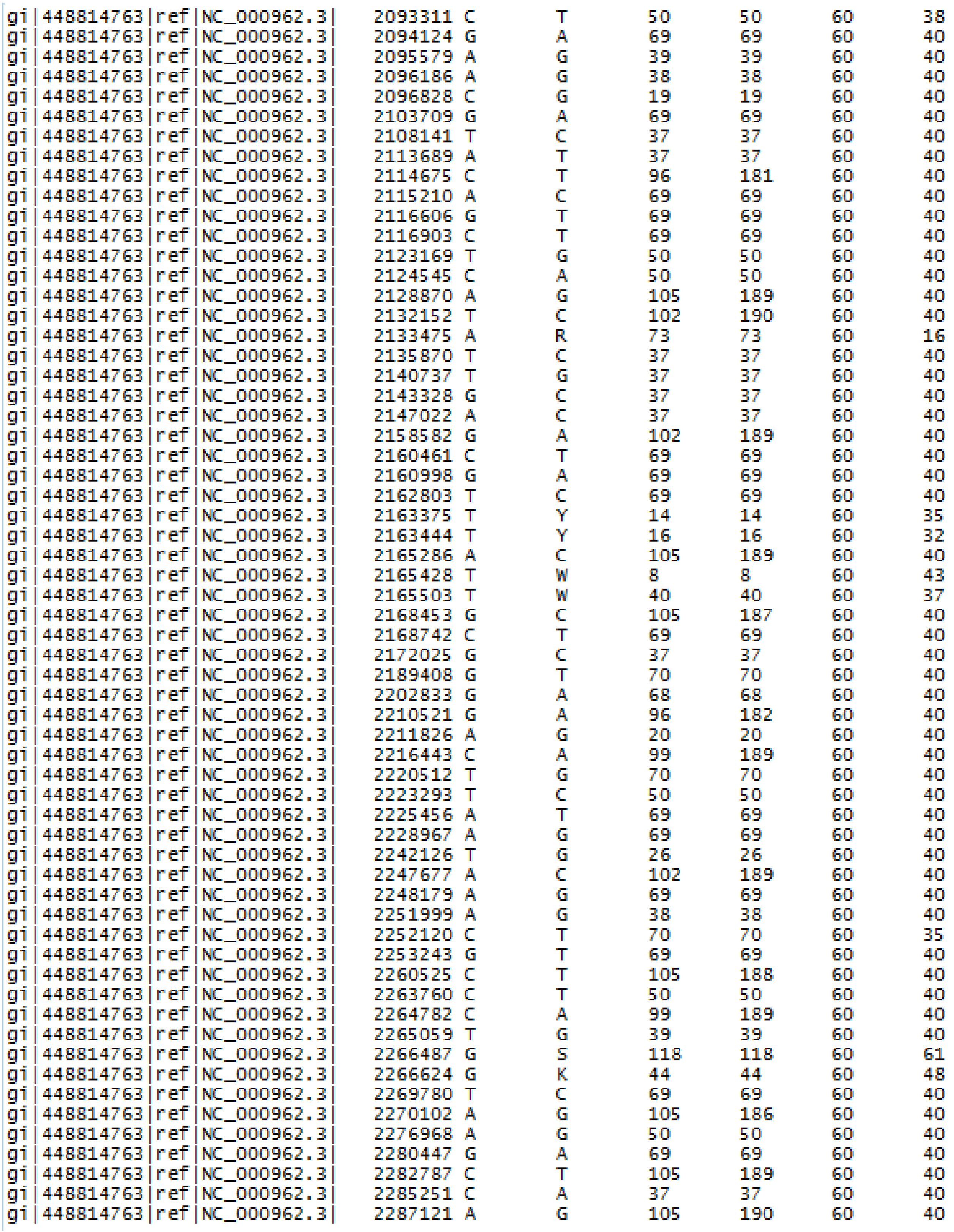

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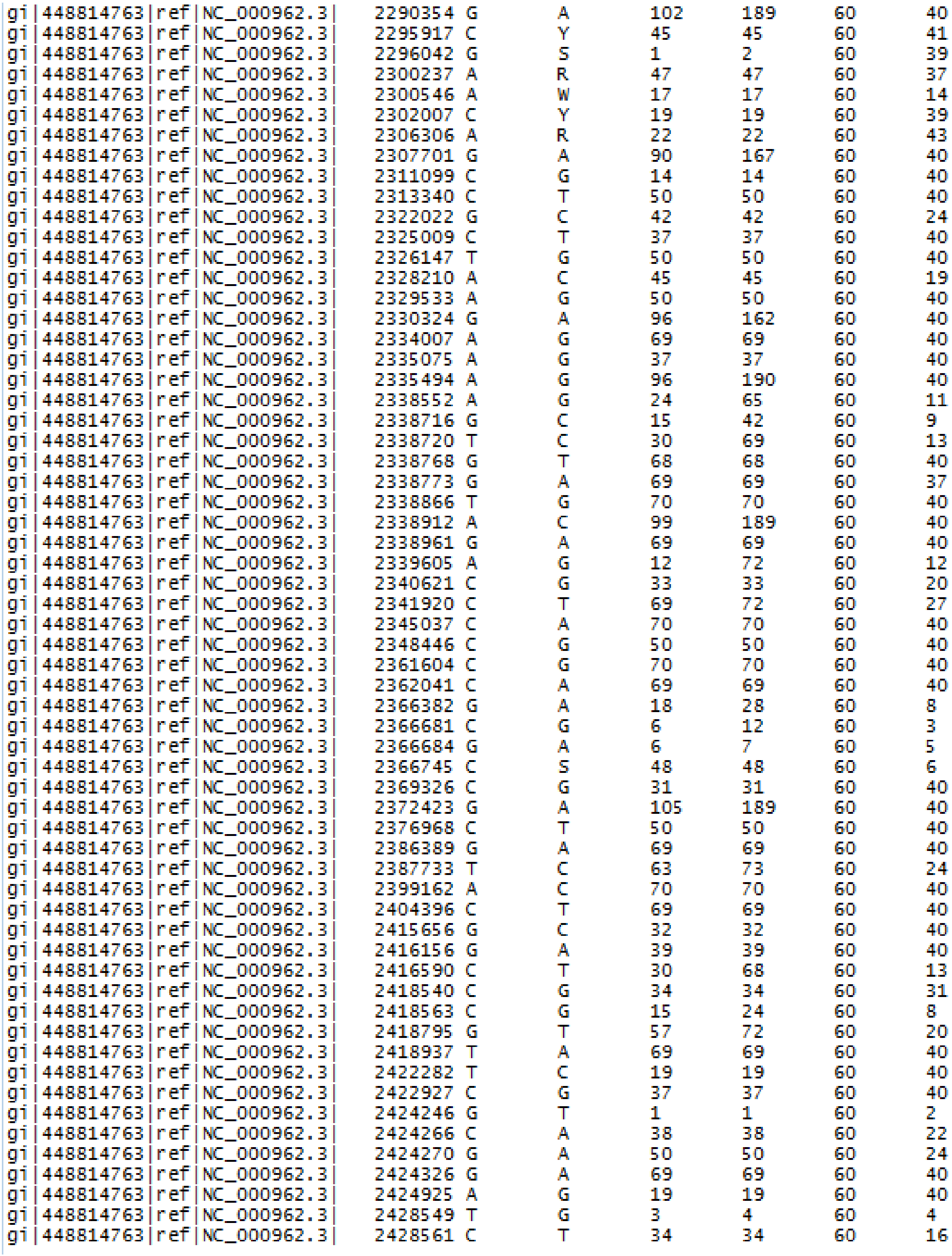

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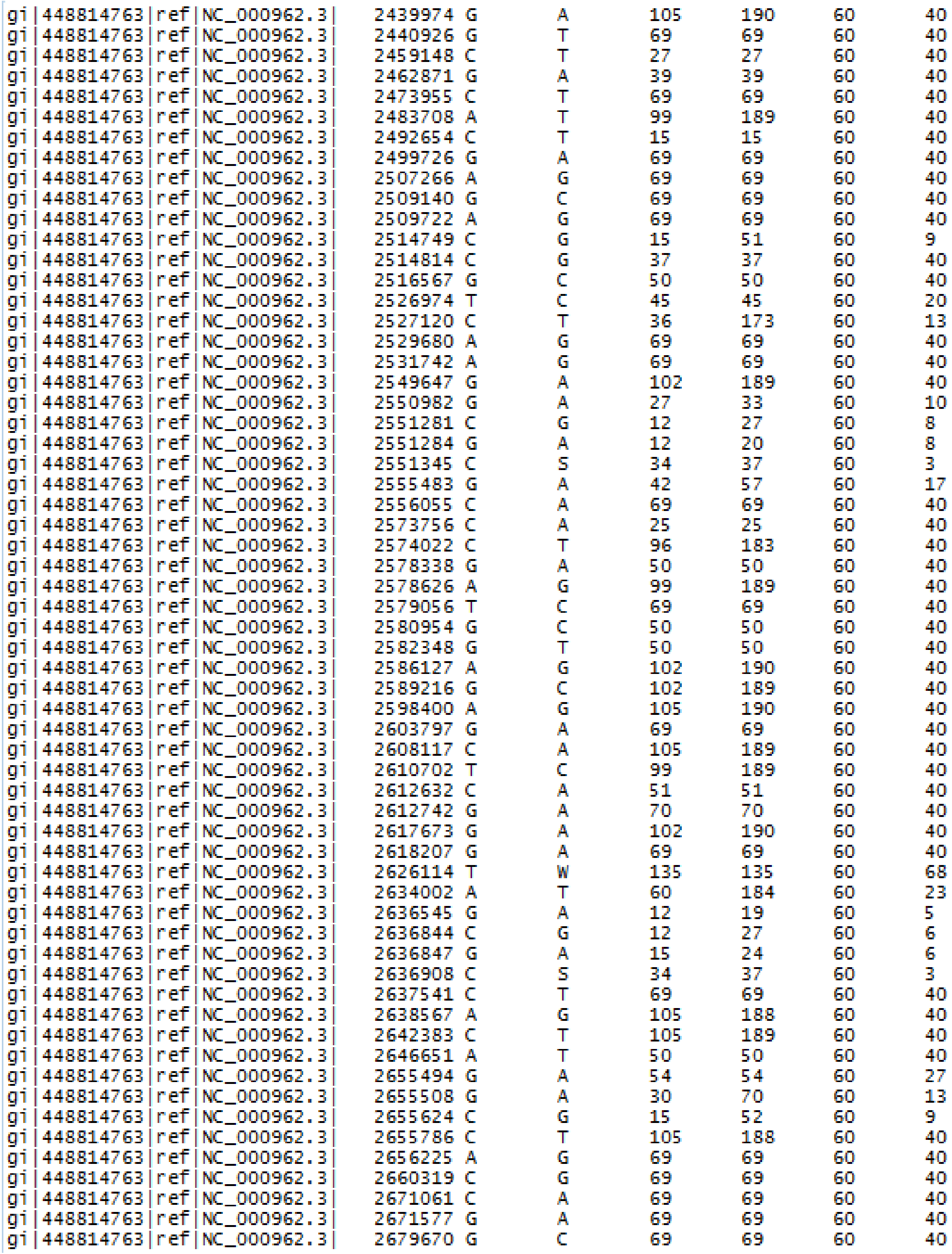

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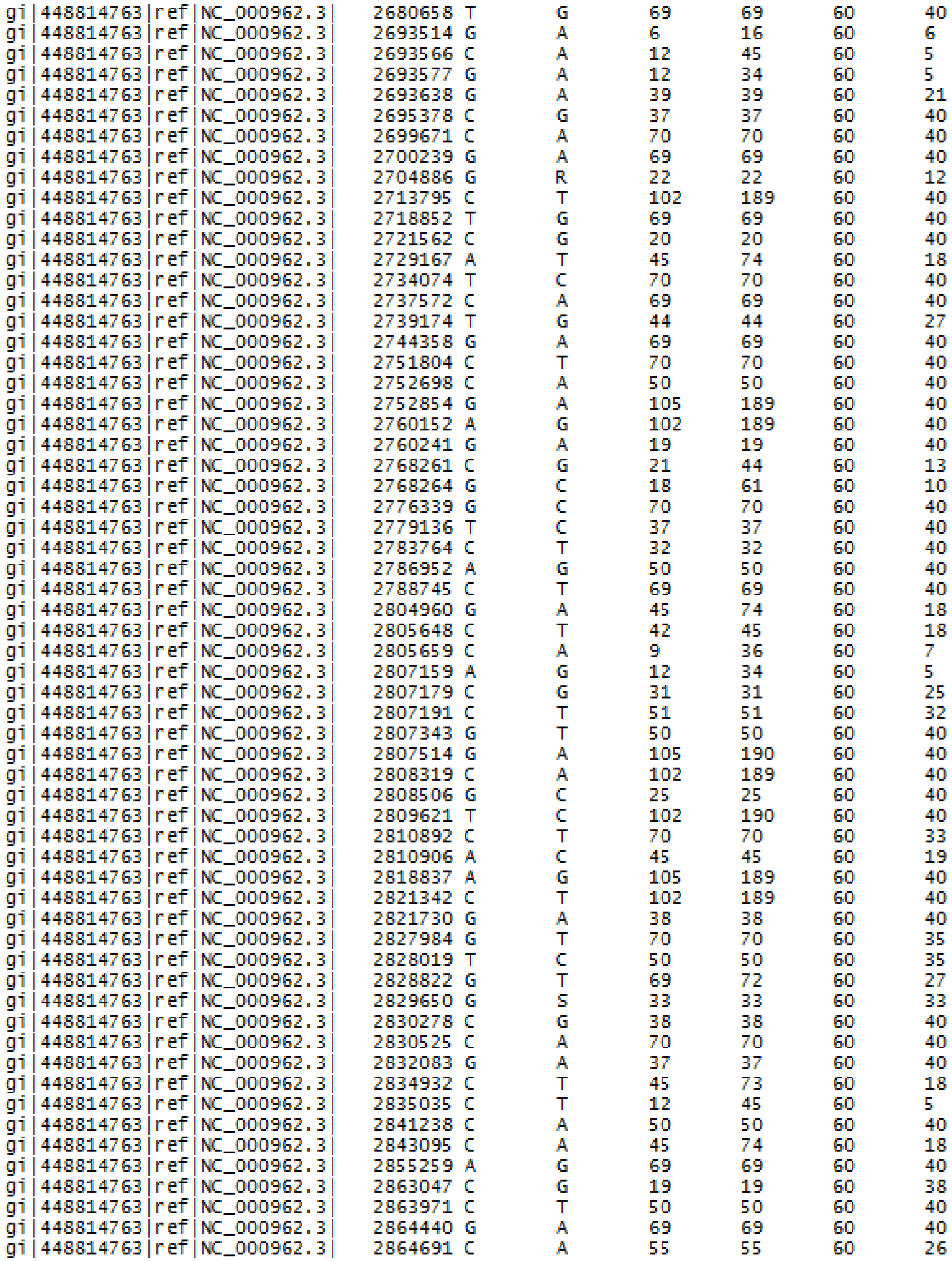

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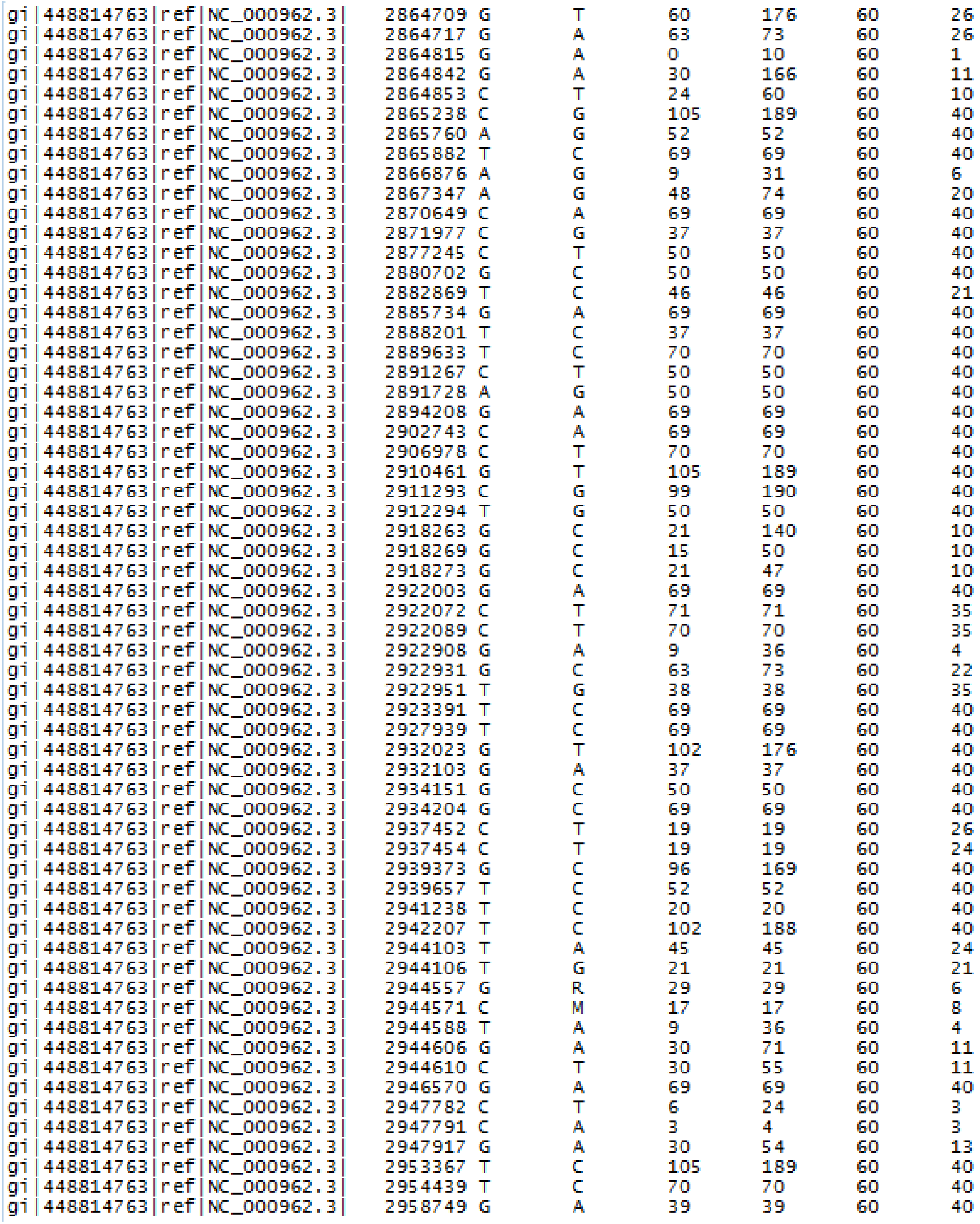

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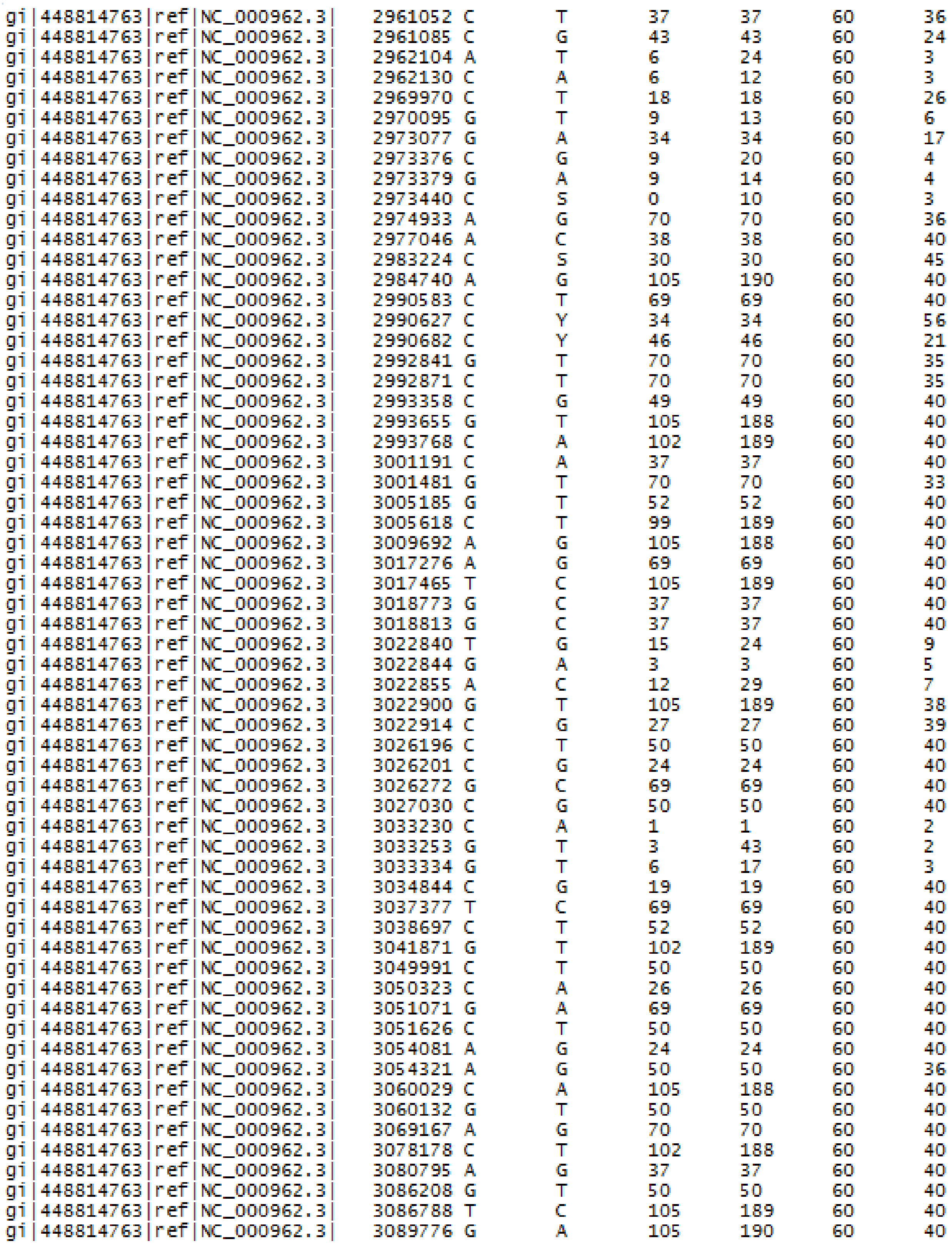

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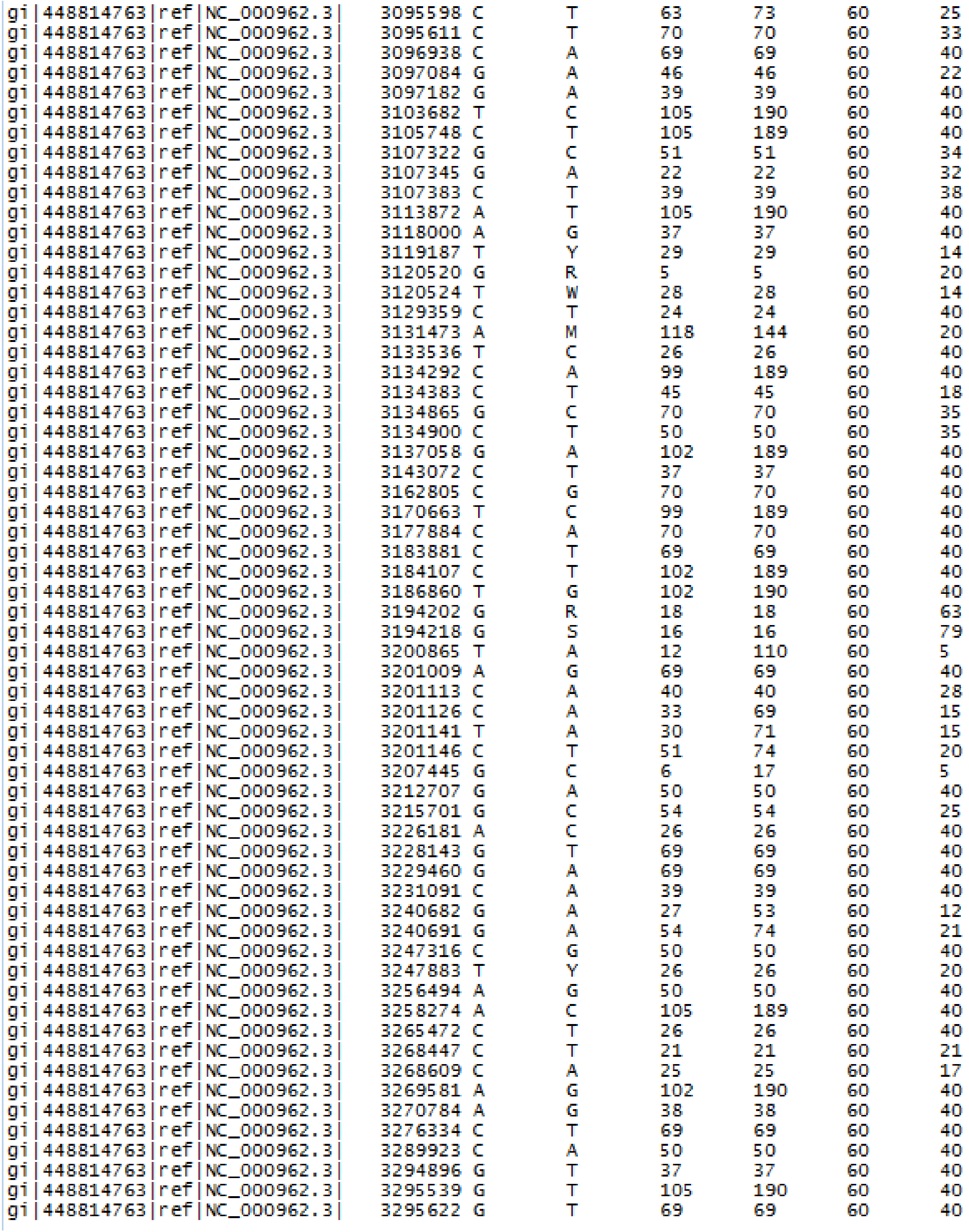

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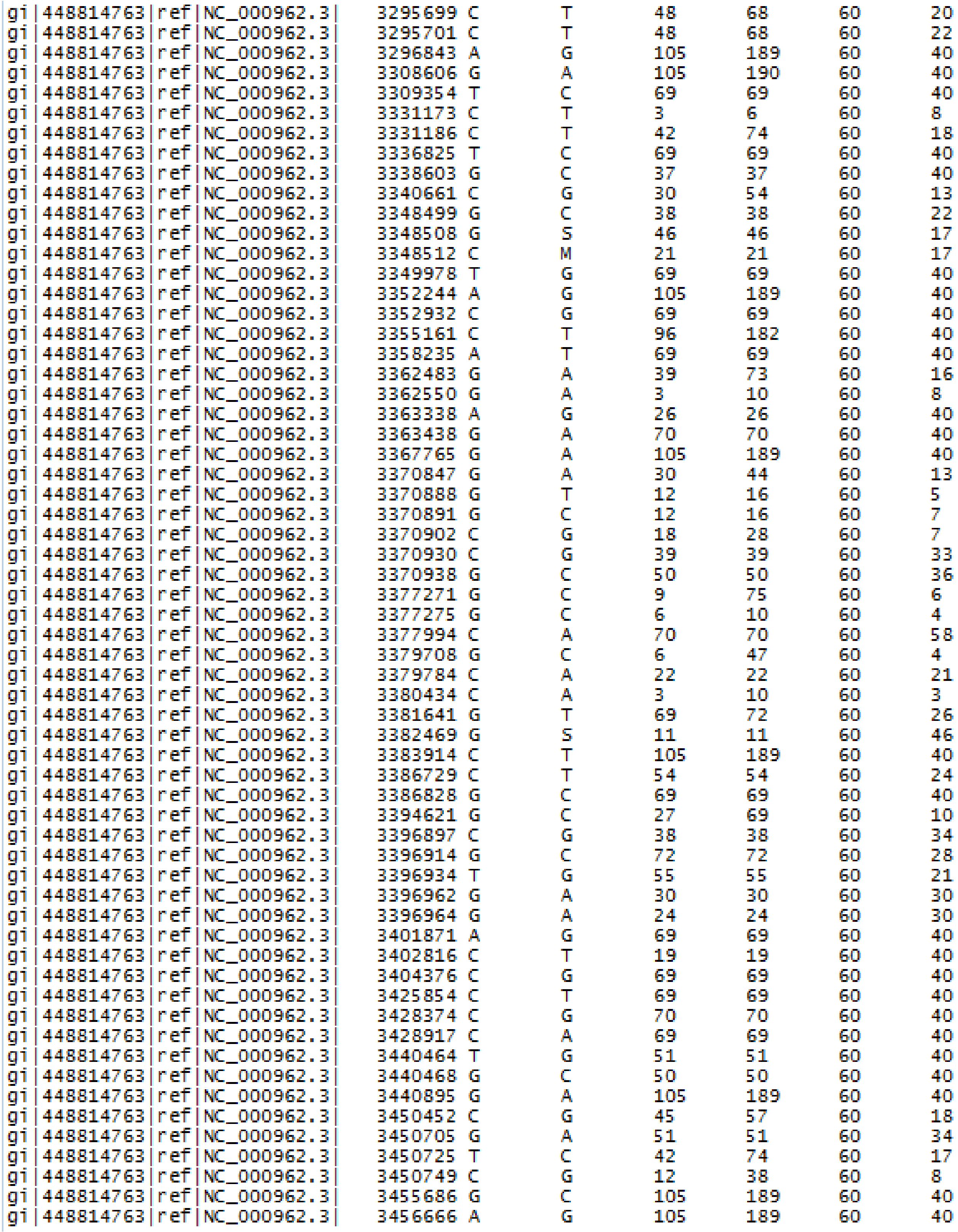

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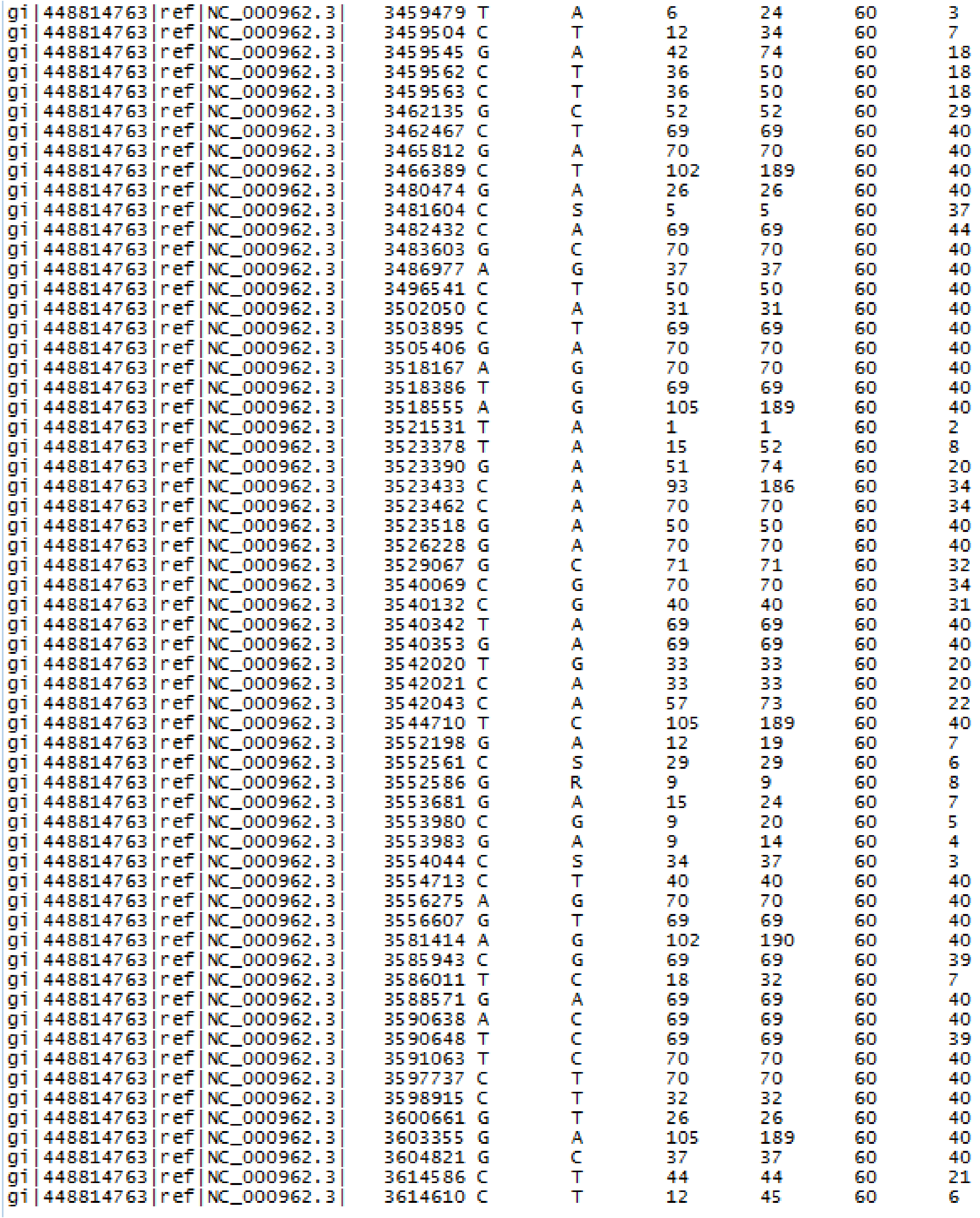

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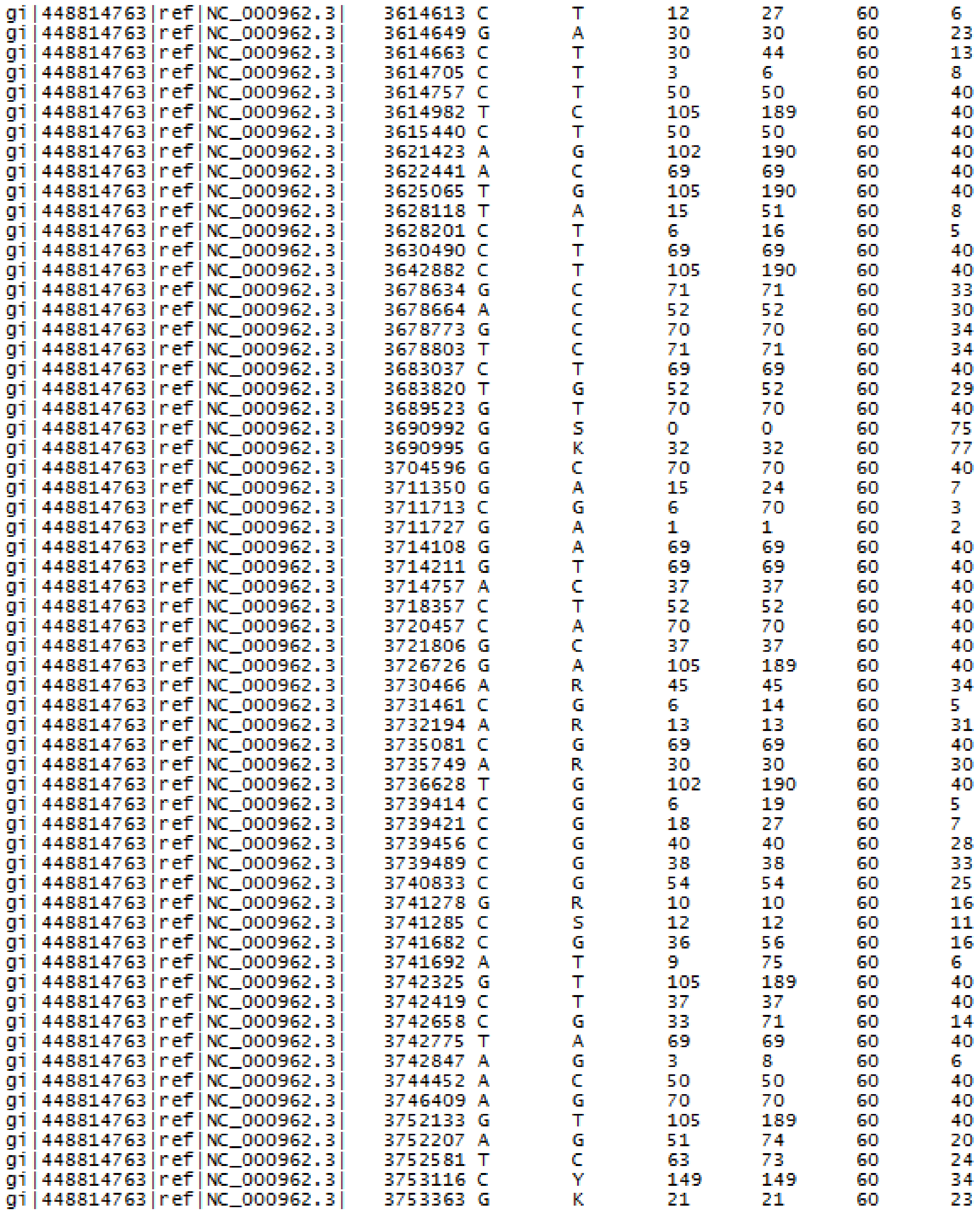

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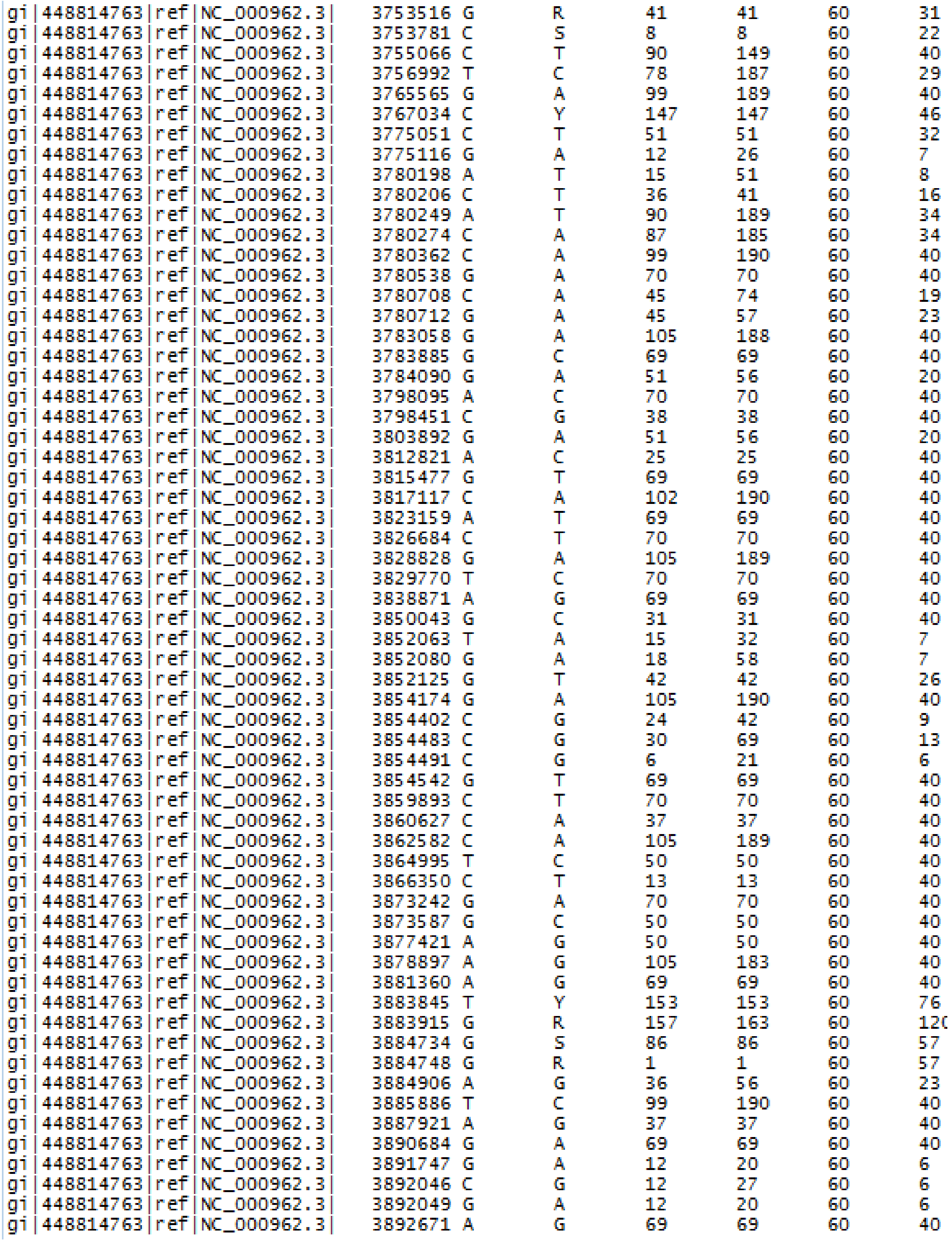

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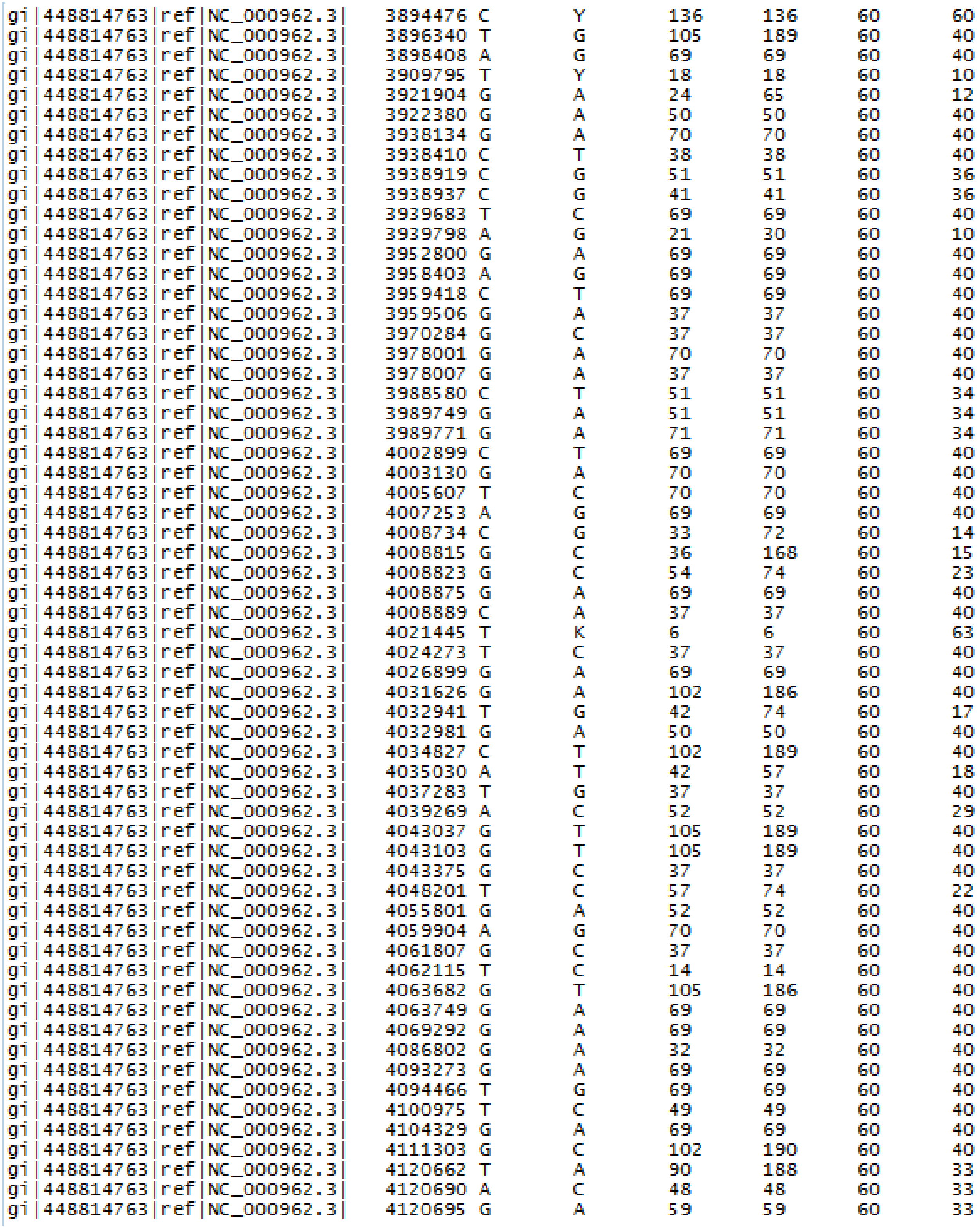

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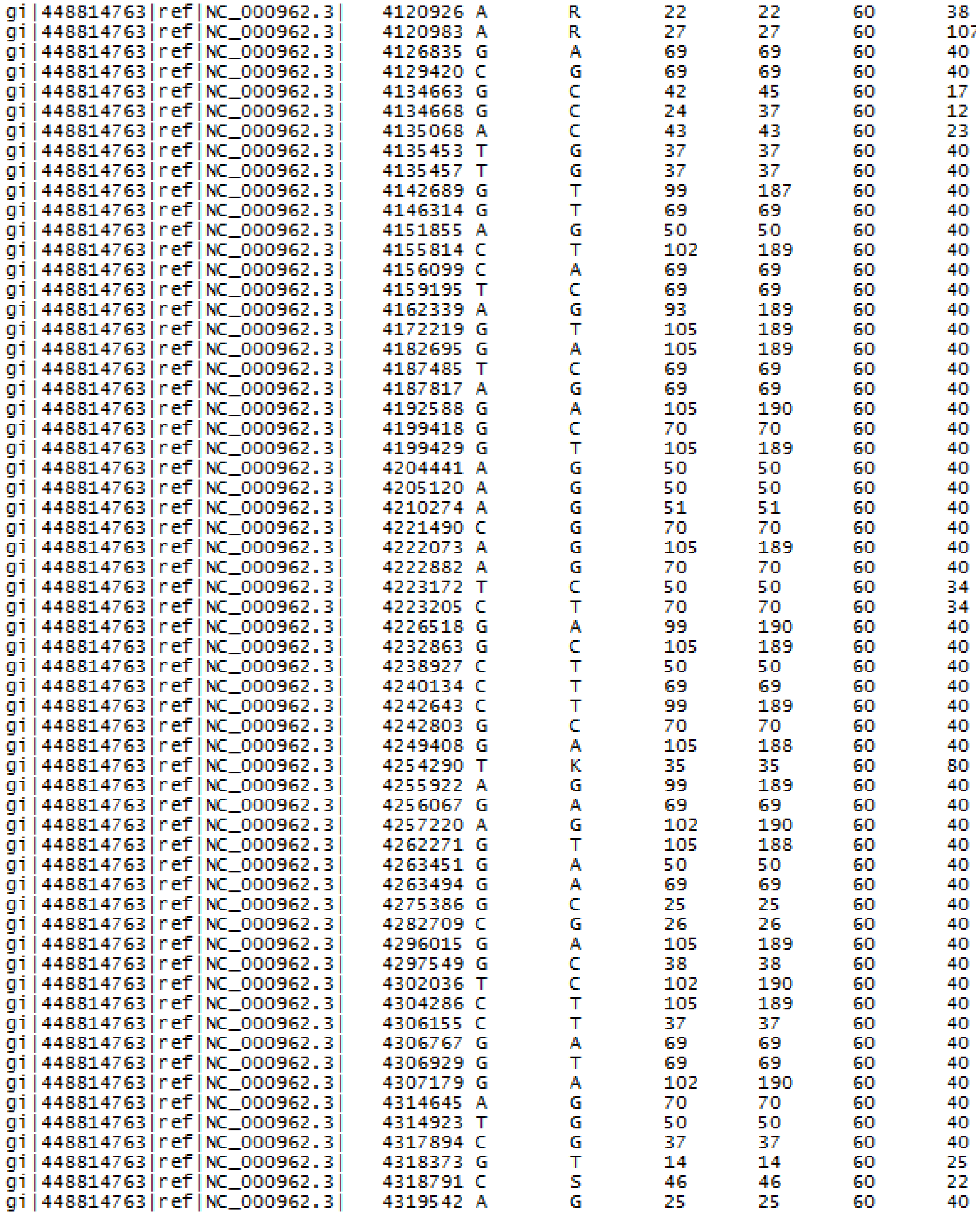

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gi|448814763|ref|NC_000962.3|	4223172 T	C	50	50	60	34
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gi|448814763|ref|NC_000962.3|	4226518 G	A	99	190	60	40
gi|448814763|ref|NC_000962.3|	4232863 G	C	105	189	60	40
gi|448814763|ref|NC_000962.3|	4238927 C	T	50	50	60	40
gi|448814763|ref|NC_000962.3|	4240134 C	T	69	69	60	40
gi|448814763|ref|NC_000962.3|	4242643 C	T	99	189	60	40
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gi|448814763|ref|NC_000962.3|	4275386 G	C	25	25	60	40
gi|448814763|ref|NC_000962.3|	4282709 C	G	26	26	60	40
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gi|448814763|ref|NC_000962.3|	4302036 T	C	102	190	60	40
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gi|448814763|ref|NC_000962.3|	4318373 G	T	14	14	60	25
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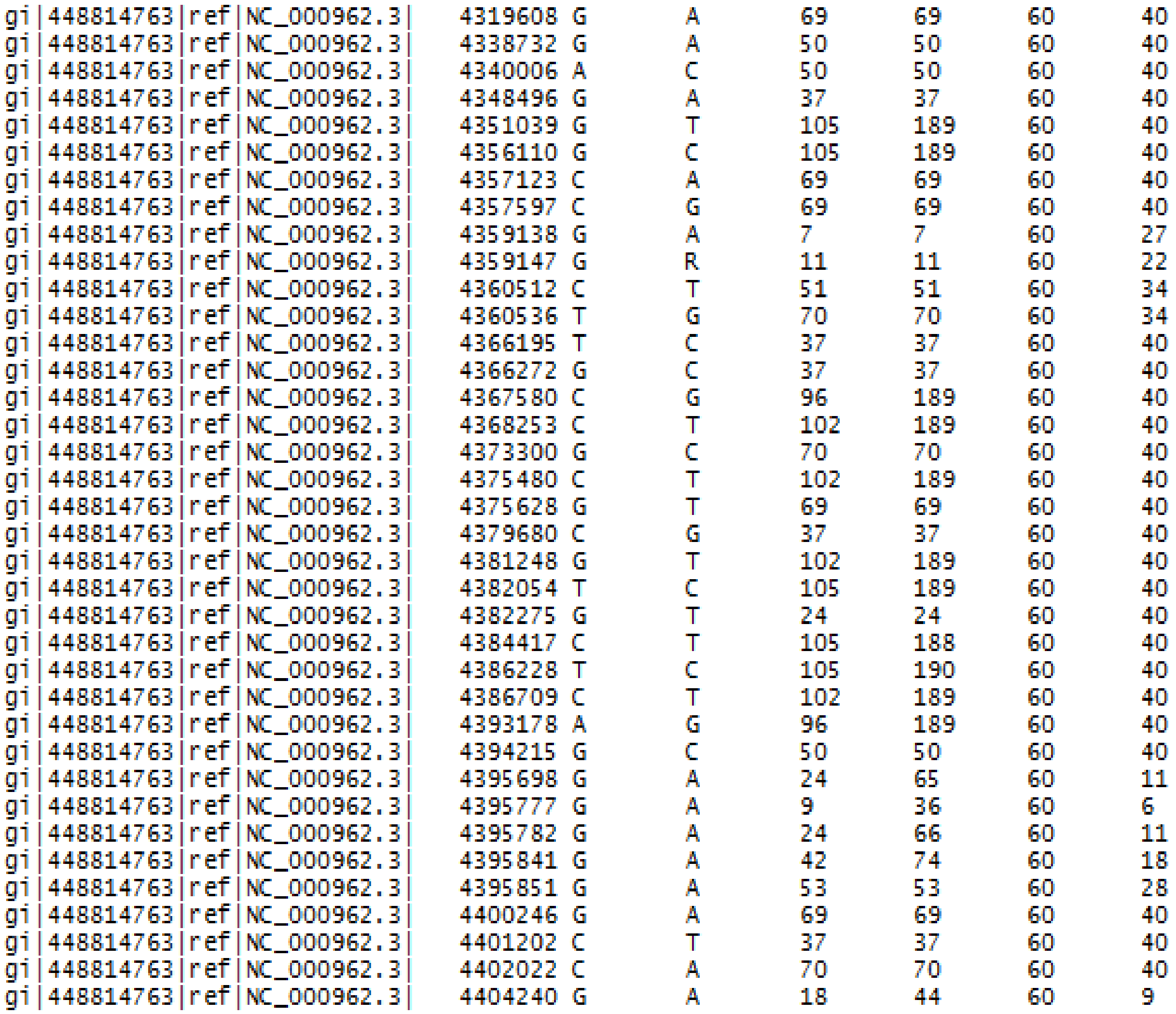

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gi|448814763|ref|NC_000962.3|    4357597 C       G       69      69      60      40
gi|448814763|ref|NC_000962.3|    4359138 G       A       7       7       60      27
gi|448814763|ref|NC_000962.3|    4359147 G       R       11      11      60      22
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