การวิเคราะห์มัลติโลคัสซีเควนซ์ของยีนในไรโซเบียมถั่วเหลืองที่แยกจากตำบลหนองกุลา จังหวัดพิษณุโลก

นางสาวเยาวภา ปุญญะฐิติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย
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MULTILOCUS SEQUENCE ANALYSIS OF GENES IN SOYBEAN RHIZOBIA ISOLATED FROM NONGKULA SUBDISTRICT, PHITSANULOK PROVINCE

Miss Yaowapa Punyathiti

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Industrial Microbiology
Department of Microbiology
Faculty of Science
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Thesis Title	MULTILOCUS SEQUENCE ANALYSIS OF GENES IN SOYBEAN RHIZOBIA ISOLATED FROM NONGKULA SUBDISTRICT, PHITSANULOK PROVINCE
By	Miss Yaowapa Punyathiti
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.....Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.rer.nat)

THESIS COMMITTEE

.....Chairman

(Associate Professor Sirirat Rengpipat, Ph.D.)

.....Thesis Advisor

(Associate Professor Kanjana Chansa-ngavej, Ph.D.)

.....Examiner

(Assistant Professor Wanchai Assavalapsakul, Ph.D.)

.....External Examiner

(Assistant Professor Wipa Homhaul, Ph.D.)

เยาวภา ปุญญะฐิติ : การวิเคราะห์มัลติโลคัสซีเควันซ์ของยีนในไรโซเบียมถั่วเหลืองที่แยกจากตำบลหนองกุลา จังหวัดพิษณุโลก. (MULTILOCUS SEQUENCE ANALYSIS OF GENES IN SOYBEAN RHIZOBIA ISOLATED FROM NONGKULA SUBDISTRICT, PHITSANULOK PROVINCE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. กาญจนา ชาญสง่าเวช, 212 หน้า.

ไรโซเบียมถั่วเหลืองเป็นแบคทีเรียในปมรากถั่วเหลืองซึ่งเปลี่ยนไนโตรเจนจากอากาศ เป็นแอมโมเนียให้ถั่วเหลืองใช้ในการเจริญ ในปัจจุบันพื้นที่เพาะปลูกถั่วเหลืองในประเทศไทยลดลงทุกปี และประเทศไทยนำเข้าถั่วเหลืองประมาณ 85% ของถั่วเหลืองที่บริโภคในประเทศ ทำให้ขาดดุลการค้าและขาดการบำรุงดินอย่างยั่งยืน วัตถุประสงค์ของงานวิจัยเพื่อจำแนกชนิดไรโซเบียมถั่วเหลืองจาก ต.หนองกุลา จ. พิษณุโลก วิธีทดลองประกอบด้วย การใช้แบคทีเรียประเภทเพิ่มจำนวนช้าที่แยกจากปมรากถั่วเหลืองพันธุ์เชียงใหม่ 2 ที่ปลูกในแปลงทดลองขนาด 15 x 24 ตารางเมตร ใน ต. หนองกุลา จ. พิษณุโลก และหาลายพิมพ์ดีเอ็นเอโดยใช้ปฏิกิริยา RAPD-PCR โดยใช้ RPO1 หรือ CRL-7 เป็นไพร์เมอร์ การสร้างต้นไม้วิวัฒนาการหรือเดนโดรแกรมจากลายพิมพ์ดีเอ็นเอและการคัดเลือกไรโซเบียมถั่วเหลืองประเภทเพิ่มจำนวนช้าจำนวน 5 สายพันธุ์ ได้แก่ NKL09216, NKL09231, NKL09273, NKL09666 และ NKL09693 เพื่อจำแนกชนิดไรโซเบียมถั่วเหลืองโดยการวิเคราะห์มัลติโลคัสซีเควันซ์ของยีน 16S rDNA, *dnaK*, *nifH*, *glnII* และ *recA* ผลการทดลองได้แบคทีเรียประเภทเพิ่มจำนวนช้าจำนวน 116 ไอโซเลต ผลการจัดไอโซเลตที่มีลายพิมพ์ดีเอ็นเอเหมือนกันเป็นสายพันธุ์เดียวกัน พบว่าได้แบคทีเรีย 43 สายพันธุ์ ซึ่งผลการทดสอบความสามารถในการสร้างปมที่รากถั่วเหลือง (*Glycine max*) พันธุ์ ชม2, ชม60, สท1, สท2, สท3, สจ4, สจ5 และ ศรีสำโรง1 พบว่าแบคทีเรียทั้ง 43 สายพันธุ์เป็นไรโซเบียมถั่วเหลือง ผลการเปรียบเทียบลำดับนิวคลีโอไทด์ของยีน 16S rDNA, *glnII,* และ *nifH* โดยใช้โปรแกรม BLAST พบว่า สามารถแบ่งไรโซเบียมถั่วเหลืองทั้ง 5 สายพันธุ์ออกเป็น 2 กลุ่มอย่างชัดเจน โดยกลุ่มแรกประกอบด้วยสายพันธุ์ NKL09216, NKL09231, NKL09666 และ NKL09693 ซึ่งอาจเป็น *Bradyrhizobium yuanmingense* ในขณะที่สายพันธุ์ NKL09273 เป็น *B.elkanii* ผลการเปรียบเทียบลำดับนิวคลีโอไทด์ของยีน *recA* โดยใช้โปรแกรม BLAST พบว่าทั้ง 5 สายพันธุ์อาจเป็น *B. japonicum* อย่างไรก็ตาม ผลการเปรียบเทียบลำดับนิวคลีโอไทด์ของยีน *dnaK* ไม่สามารถจำแนกสายพันธุ์ของไรโซเบียมถั่วเหลืองทั้ง 5 สายพันธุ์ ผลการสร้างเดนโดรแกรมโดยใช้สายพันธุ์อ้างอิง 16-19 สายพันธุ์ พบว่า เฉพาะเดนโดรแกรมที่สร้างจากลำดับนิวคลีโอไทด์ของยีน *glnII* ให้ผลการจำแนกชนิดเช่นเดียวกับที่ใช้วิธีเปรียบเทียบลำดับนิวคลีโอไทด์ของยีนแต่ละยีนโดยใช้โปรแกรม BLAST ส่วนผลการสร้างเดนโดรแกรมจากลำดับนิวคลีโอไทด์ของยีน 16S rDNA, *dnaK*, *nifH*, *recA* และลำดับนิวคลีโอไทด์ของยีนทั้งห้ายีนซึ่งนำมาเรียงต่อกัน ไม่สามารถจำแนกชนิดของไรโซเบียมถั่วเหลืองทั้ง 5 สายพันธุ์ เพราะผลการทดลองพบว่าไรโซเบียมถั่วเหลืองทั้ง 5 สายพันธุ์มีความใกล้ชิดทางวิวัฒนาการกับ *Bradyrhizobium elkanii, B. japonicum*, *B. liaoningense*, และ *B. yuanmingense.*

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2556

5372535023 : MAJOR INDUSTRIAL MICROBIOLOGY
KEYWORDS: GENETIC DIVERSITY /SOYBEAN RHIZOBIUM /NONGKULA SUBDISTRICT.

YAOWAPA PUNYATHITI: MULTILOCUS SEQUENCE ANALYSIS OF GENES IN SOYBEAN RHIZOBIA ISOLATED FROM NONGKULA SUBDISTRICT, PHITSANULOK PROVINCE. ADVISOR: ASSOC. PROF. KANJANA CHANSA-NGAVEJ, 212 pp.

Soybean rhizobia are bacteria in soybean root nodules which are able to convert atmospheric nitrogen to ammonia for soybeans to assimilate for growth. At present, there has been an annual decline in soybean cultivation areas and Thailand imports approximately 85% of local soybean consumption resulting in a trade deficit and in an opportunity loss for sustainable maintenance of soil quality. The aims of this research were to identify slow-growing soybean rhizobia from root nodules of soybean cultivar Chiangmai 2 grown in a 15 x 24 sq.m. experimental plot in Nongkula subdistrict, Phitsanulok province. Methods included RAPD-PCR fingerprinting with either RPO1 or CRL-7 as the primer, grouping slow-growing bacterial isolates with identical RAPD-PCR fingerprints into the same strains, constructing dendrograms from RAPD-PCR fingerprints, and identification of 5 selected soybean rhizobia by Multilocus Sequence Analysis (MLSA) of 16S rDNA, *dnaK*, *nifH*, *glnII* and *recA*. Experimental results showed 116 slow-growing bacterial isolates were obtained. Identical RAPD-PCR fingerprints showed 116 slow-growing bacterial isolates were 43 strains. Authentication tests with soybean seeds (*Glycine max* cv. CM2, CM60, ST1, ST2, ST3, SJ4, SJ5 and Sri Samrong1) revealed all the 43 strains were soybean rhizobia. BLAST results of *glnII* revealed the 5 soybean rhizobial strains could be grouped into two groups with strains NKL09216, NKL09231, NKL09666 and NKL09693 were found to be *Bradyrhizobium yuanmingense* while strain NKL09273 was found to be *B. elkanii*. MLSA using *glnII* yielded the same results as obtained from the BLAST program while MLSA from dendrograms constructed from sequences of the remaining four genes and concatenated sequences of the 5 genes could not identify the 5 soybean rhizobial strains into different species. They were found to be related to *Bradyrhizobium elkanii, B. japonicum*, *B. liaoningense*, and *B. yuanmingense.*

Department: Microbiology Student's Signature .....

Field of Study: Industrial Microbiology Advisor's Signature .....

Academic Year: 2013

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Special thanks are expressed to friends and student members in Laboratory 1904/13, and all staff members of the Department of Microbiology for their help and friendship during my study.

The last, but most important, is my sincere and deepest gratitude to my parents and every member in my family for their great love, constant support, understanding and heartfelt encouragement extended throughout my study.

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CHAPTER I
INTRODUCTION

1. Soybean rhizobia

Soybean rhizobia are Gram negative, rod-shaped non-spore forming, motile bacteria which fix nitrogen in root nodules of soybean *Glycine max* (L.) There are two categories of soybean rhizobia : Fast-growing soybean rhizobia and slow-growing soybean rhizobia. At present, six species of soybean rhizobia are recognized as follows:

Fast-growers :

Sinorhizobium fredii (Chen et al., 1988)

Sinorhizobium xinjiangense (Peng et al., 2002)

Slow-growers :

Bradyrhizobium elkanii (Kuykendall et al., 1992)

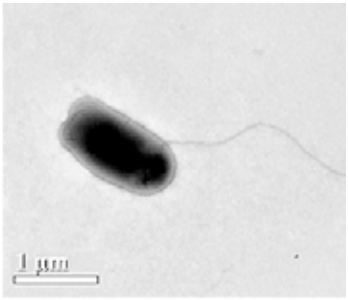
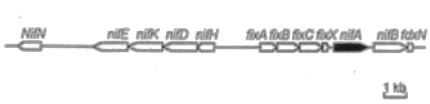
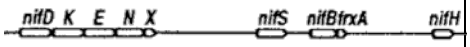
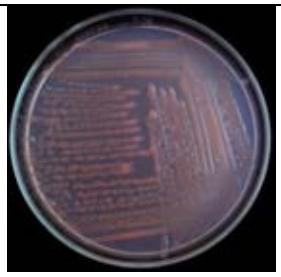
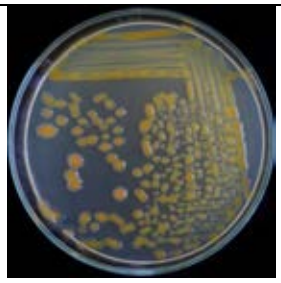
Bradyrhizobium japonicum (Jordan, 1982)

Bradyrhizobium liaoningense (Xu et al., 1995)

Bradyrhizobium yuanmingense (Appune et al., 2008)

Some differences between fast-and slow-growing soybean rhizobia are shown in Table 1.1.

Table 1.1 Some differences between fast- and slow-growing soybean rhizobia (Elkan & Bunn, 1992; this study).

Properties	Soybean rhizobia	
	Fast-growers	**Slow-growers**
1. Doubling time	Less than 6 hours	More than 6 hours
2. Type and number of flagella	2-6 peritrichous flagella 	1 subpolar flagellum 
3. *nifHDK*	*nifHDK* are in the same operon 	*nifH* and *nifDK* are on separate operons 
4.Colony morphology	 NKL09114	 STB8 *B.elkanii*
5.Bromothymol blue (BTB) reactions	 NKL09114	 STB8 *B.elkanii*

So far, considerable amounts of research have been conducted in Thailand and other leading soybean exporting countries, notably the US, People's Republic of China, Argentina, and Brazil, on the isolation and characterization of soybean rhizobia. However , despite great diversity of soybean rhizobia including different abilities to secrete either acidic or alkali products (Bromothymol Blue reactions), different abilities to utilize carbon and nitrogen sources, different patterns of growth at different temperatures, different RAPD-PCR fingerprints, polyphasic taxonomy including the use of 16S rDNA sequences to identify isolated soybean rhizobia strains only revealed 4 different species of slow-growing soybean rhizobia worldwide. One reason for the recognition of only 4 species is because 16S rDNA sequences which are conserved are mostly used in the identification process. This practice hinders the progress in soybean rhizobial taxonomy. Therefore, this thesis aims to employ Multilocus Sequence Analysis using sequences of four housekeeping genes and one symbiotic gene, namely 16S rDNA, *dnaK*, *glnII*, *recA* and *nifH*, respectively, to identify and determine phylogenetic relationships amongst soybean rhizobia isolated from Nongkula subdistrict, Phitsanulok province.

CHAPTER II
LITERATURE SURVEY

2.1 Research work on identification of soybean rhizobia in Thailand

Research on identification of soybean rhizobia in Thailand is not as extensive as those conducted in soybean exporting countries such as USA, Brazil, and Argentina(http://www.rizobacter.com.ar/risoja.html,http://www.americasbestinoculant.com/,http://www.beckerunderwood.com/en/inoculants), Appunu et al., 2008; Menna et al., 2006). Research work on the characterization by 16S rDNA sequences of soybean rhizobia in Nan and Phitsanulok provinces has shown that only slow-growing soybean rhizobia *Bradyrhizobium elkanii, B. japonicum, B. liaoningense* and *B. yuanmingense* have been isolated from Thailand (Ando and Yokoyama, 1999; Chanthapetch, 2009; Maruekarajtinplaeng, 2010). In 2012 Maruekarajtinplaeng et al., isolated soybean rhizobia from 16 subdistricts of Phitsanulok province and used polyphasic taxonomy including the use of 16S rDNA sequences to identify *Bradyrhizobium* spp. The researchers reported the detection of *B. yuanmingense* for the first time in Thailand. In addition, the researchers found that the identified *B. elkanii* strains STB8, STB119, STB120, STB147, STB173, STB220, and STB245 had different RAPD-PCR fingerprints when the arbitrarily GC rich CRL-7 was used as the primer. However, the 7 *B. elkanii* strains were found to have different abilities to secrete either acidic or alkali products, different abilities to utilize 95 different carbon and nitrogen compounds, different patterns of growth at different temperatures. Therefore, it was suggested that the 7 strains were natural variants of *B. elkanii.* Similarly, strains STB30, STB54, STB67, STB96, STB250, and STB310 were found to be natural variants of *B. japonicum*. Hence, the use of polyphasic taxonomy as described by Vandamme et al. (1996) is not sufficient to refine the identification of slow-growing soybean rhizobia up to either the species or strain levels. Slow-growing soybean rhizobia might contain more than the 4 species presently recognized worldwide which are *Bradyrhizobium elkanii (*Kuykendall et al., 1992*), B. japonicum (*Jordan, 1982*), B. liaoningense* (Xu et al., 1995), and *B. yuanmingense* (Appunu et al., 2008). In this thesis, Multilocus Sequence Analysis (MLSA) as described by Gevers et al. (2005) will be employed to identify soybean rhizobia for the first time in Thailand

by using sequences of the following 4 house-keeping genes and one symbiotic gene, respectively: 16S rDNA, *dnaK, glnII, recA,* and *nifH.* All these genes have been used extensively in Multilocus Sequence Analysis of rhizobia (Menna et al., 2009; Ribeiro et al., 2009; Vinuesa et al., 2008). The following section describes properties of some of the genes used in this thesis.

Soybeans are grown as rotational crop in rotation with economic plants such as rice, corn and sugarcane in the northern, northeastern, upper central and eastern parts of Thailand as shown in Figure 2.1

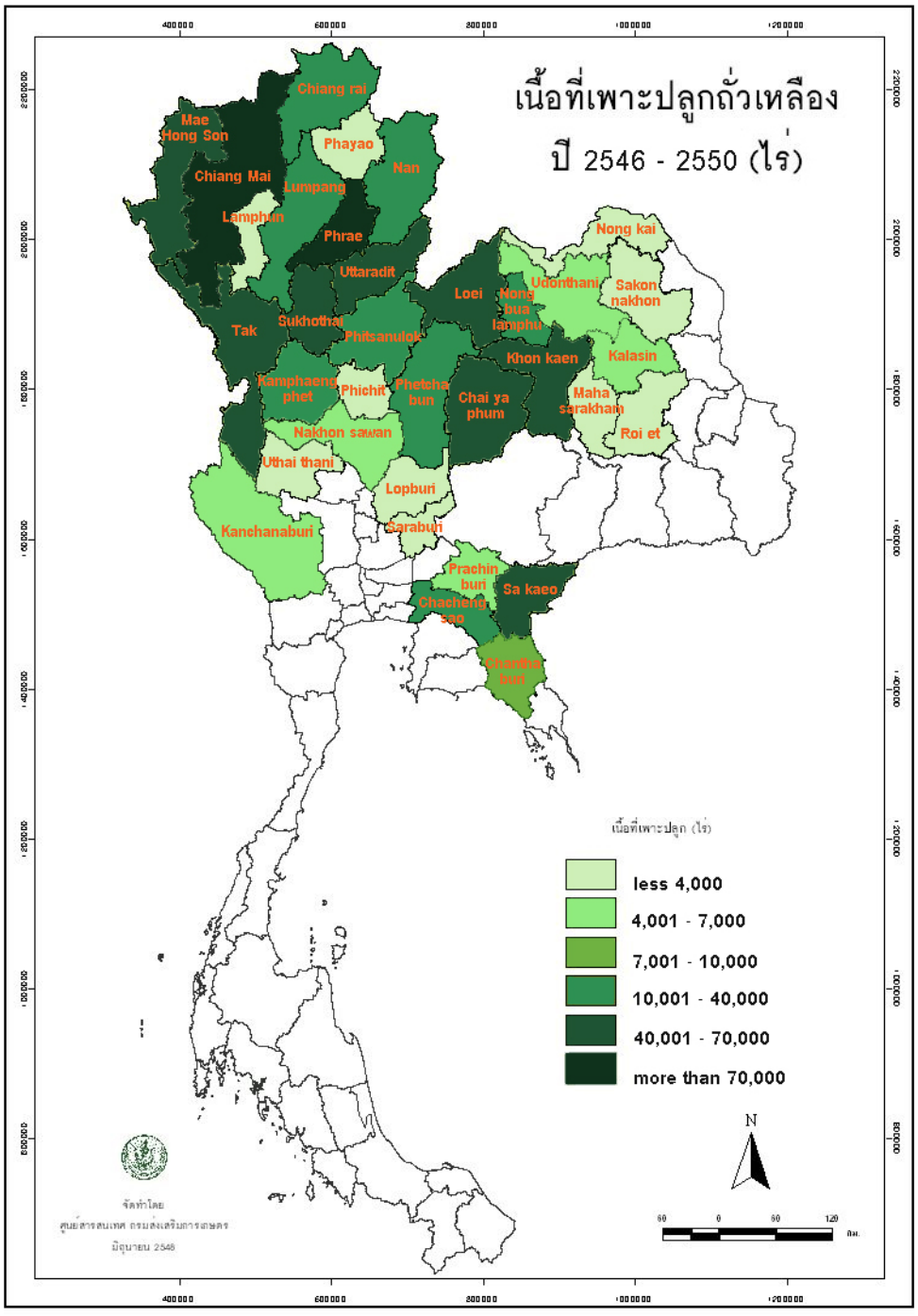


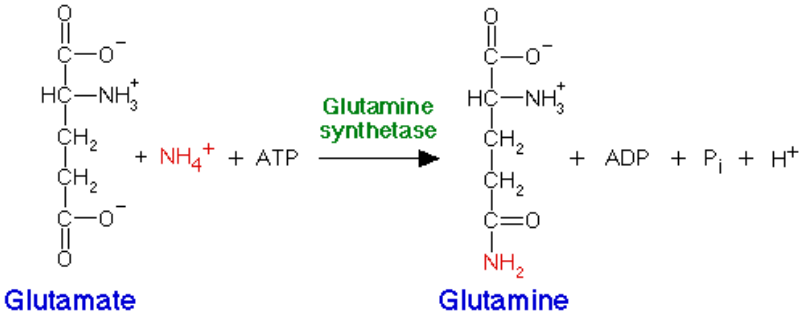
Figure 2.1 Map of Thailand showing different areas of soybean cultivation (Source: Statistics on Agriculture in Thailand in the years 2003 to 2007. Office of Agricultural Economics).

Soybean rhizobia are Gram negative bacteria which fix nitrogen in root nodules of soybeans as shown in the following equation:

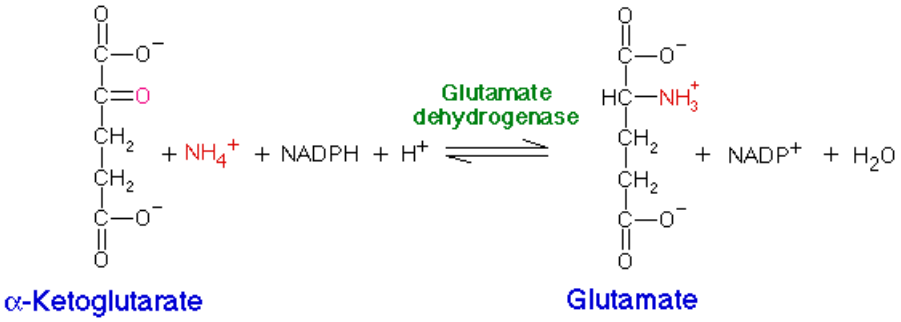
$$N_2 + 8H^+ + 8e^- + 16ATP \xrightarrow{\text{nitrogenase}} 2NH_3 + H_2 + 16\,(ADP + PO_4^{\equiv})$$

The breakdown of the triple bond in the atmospheric nitrogen molecule is energy-intensive. The 16 ATP molecules used in fixing one molecule of atmospheric nitrogen to two molecules of ammonia are supplied by soybeans. Soybean rhizobia inhabit soybean roots symbiotically with energy supplied by soybeans and ammonia obtained from nitrogen fixation by soybean rhizobia is utilized by soybeans in the synthesis of amino acids such as Glutamine, Glutamic acid, Alanine, and Aspartic acid. Two equations in the utilization of the ammonium molecule in the production of Glutamine and Glutamate are shown in Equations (1) and (2) respectively.

Equation 1:



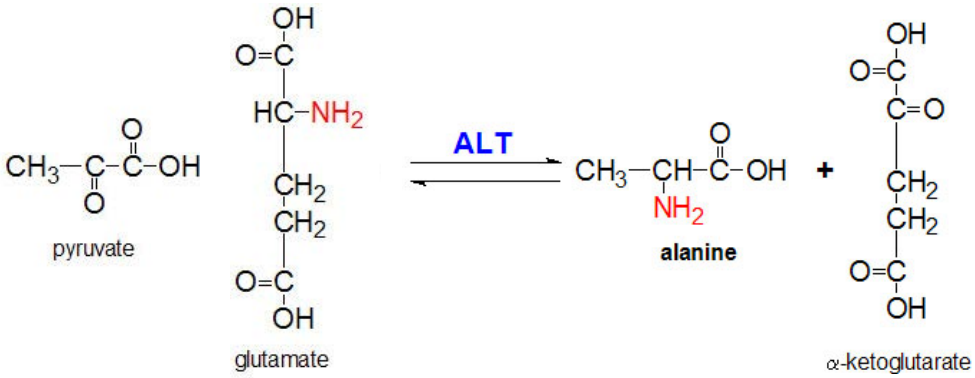
Equation 2:

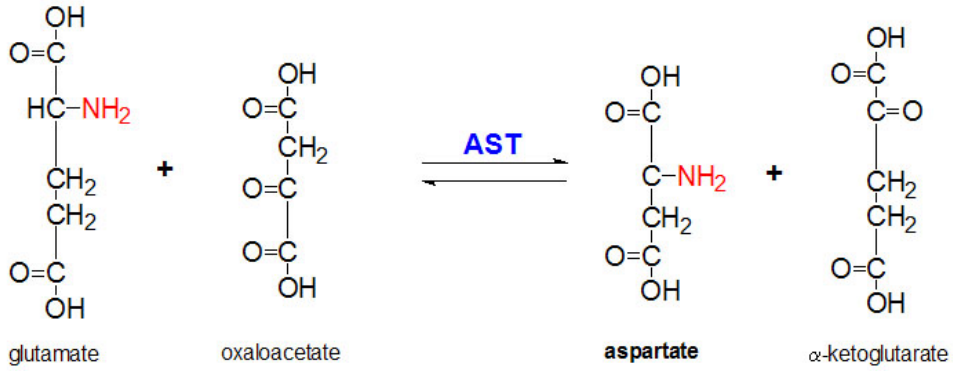


http://www.dinatec.com/Dinodornox.htm

In addition, other amino acids such as Alanine and Aspartic acid are synthesized by transamination reactions with the general equation as follows:

$$\alpha\text{-keto acid}_1 + \text{amino-acid}_1 \xrightarrow{\text{amino transferase}} \text{amino acid}_2 + \alpha\text{-keto acid}_2$$





http://themedicalbiochemistrypage.org/amino-acid-metabolism.html

The genes encoding the enzymes Glutamine synthetase and Glutamate dehydrogenase are *gln*II and *gdh* respectively.

Another gene commonly used in Multilocus Sequence Analysis (MLSA) is *nifH* which encodes the Fe protein of nitrogenase.

According to Voet and Voet (1995), The enzyme nitrogenase consists of two proteins, the Fe protein and the MoFe protein. *nifH* encodes the Fe protein which is a dimer of approximately 60 kDa which contains binding sites for ATP. *nifD* and *nifK* encode the α and β subunits of the MoFe protein which is approximately 220 kDa of subunit structure $\alpha_2\beta_2$ that contains the binding site for the substrate N_2. The overall process of nitrogen fixation in a bacterioid of rhizobia is shown in Figure 2.2

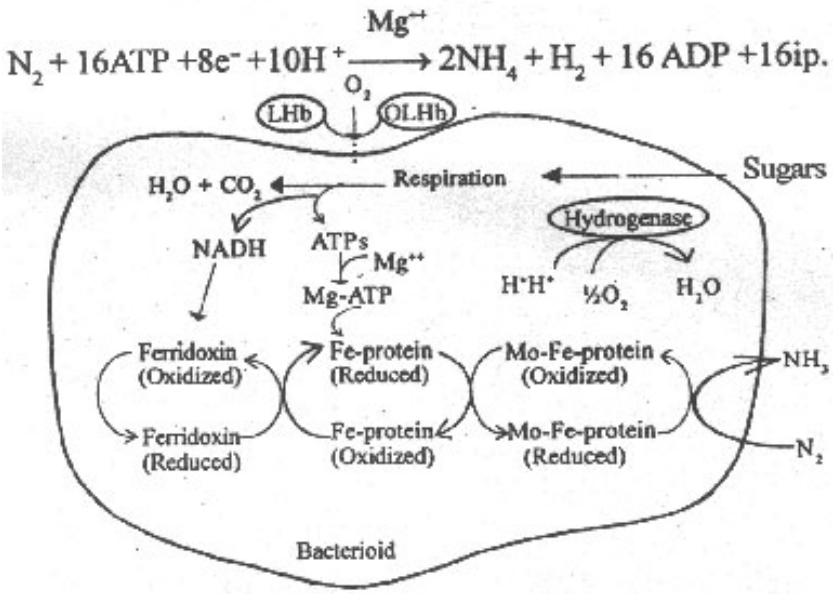


Figure 2.2 The overall process of nitrogen fixation in a bacterioid of rhizobia.

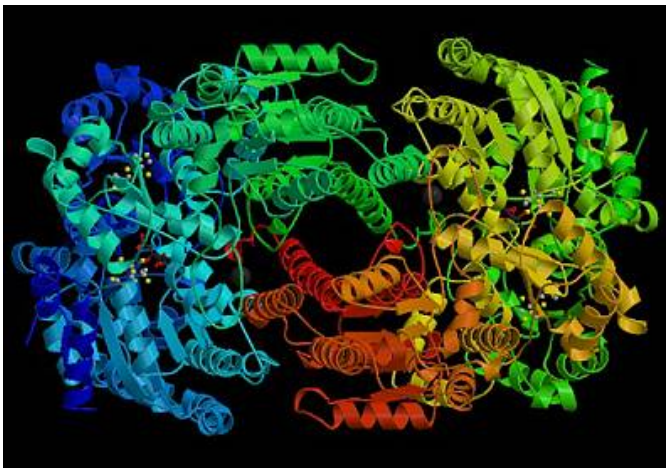


Figure 2.3 Ribbon structure of the nitrogenase MoFe protein from *Azotobacter vinelandii*.

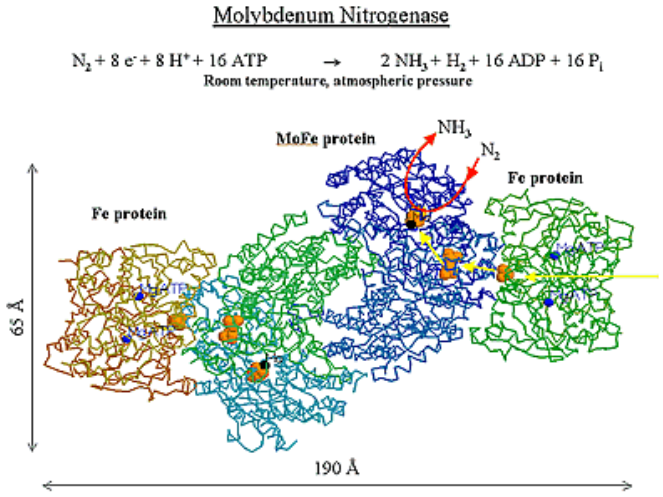


Figure 2.4 MoFe protein-Fe protein complex involved in nitrogen conversion to ammonia.

http://www.chem.cmu.edu/groups/achim/research/magneto.html

Figures 2.3 and 2.4 show ribbon structures of the nitrogenase MoFe protein and MoFe protein-Fe protein complex involved in nitrogen conversion to ammonia. Figure 2.5 shows arrangement of the five genes used in MLSA in this thesis.

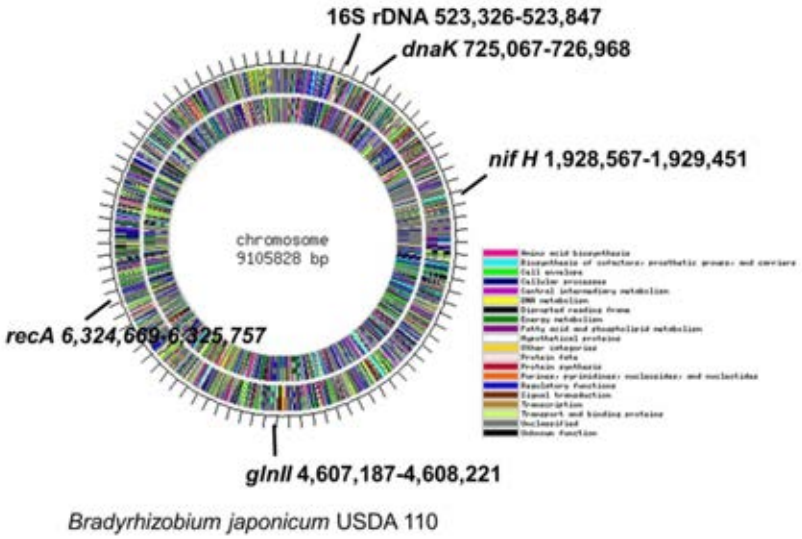


Figure 2.5 Whole genome of the slow-growing soybean rhizobium *B. japonicum* USDA110 (Kaneko et al., 2002) showing positions of the five genes proposed for use in Multilocus Sequence Analysis. All the genes in the genome are color-coded according to their functions.

recA encodes RecA enzyme which functions in homologous recombination. When a double-stranded DNA is nicked at the site where homologous recombination occurs, monomers of RecA will polymerize to form a filament with one set of sites attaches to the resultant single-stranded DNA and another set of sites attaches to the double-stranded DNA. Hence, a filament of RecA polymer surrounds both the single- and double-stranded DNA for DNA repair to take place according to complementary base pairing reaction. Figure 2.6 shows ribbon structure of a RecA monomer (http://www.callutheran.edu/BioDev/omm/reca/recamast.htm). The monomer is approximately 38 kDa.

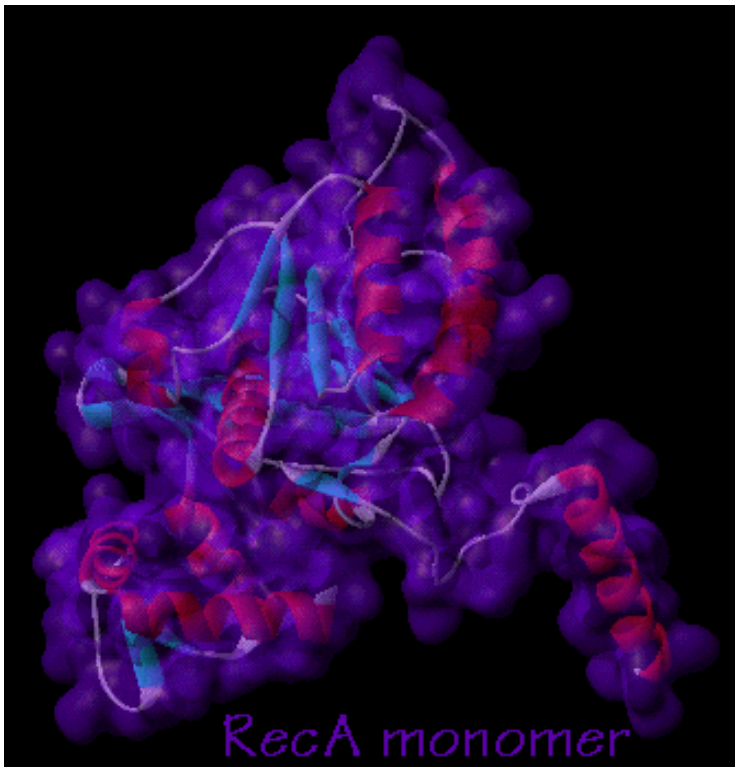


Figure 2.6 Ribbon structure of a RecA monomer.
(http://www.callutheran.edu/BioDev/omm/reca/recamast.htm).

dnaK encodes an approximately 70 kDa heat shock protein which is a molecular chaperone in all organisms including *Bradyrhizobium* spp. Figure 2.7 shows the protein product of *dnaK* consists of three domains : the ATP-binding domain of approximately 358 amino acids, the peptide-binding domain of approximately 225 amino acids and the GC- rich region of approximately 33 amino acids. Under physiological temperature, DnaK, DnaJ, GrpE, and Sigma 32 form a complex. However, under heat shock conditions, the complex separates into DnaK, DnaJ, and GrpE which function as molecular chaperones by binding to partially-denatured proteins to prevent formation of aggregates. When heat shock conditions are removed, the molecular chaperones dissociate from the partially-denatured proteins so the latter could fold back to their functioning conformation. During heat stress, Sigma 32 binds to the core enzyme of RNA polymerase to form the holoenzyme which binds to -10/-35 promoters for the transcription of genes of other heat shock proteins such as GroESL1 which aids in protein folding during heat stress as shown in Figure 2.8 (Chansa-ngavej, 2005 ; Minder et al, 1997).

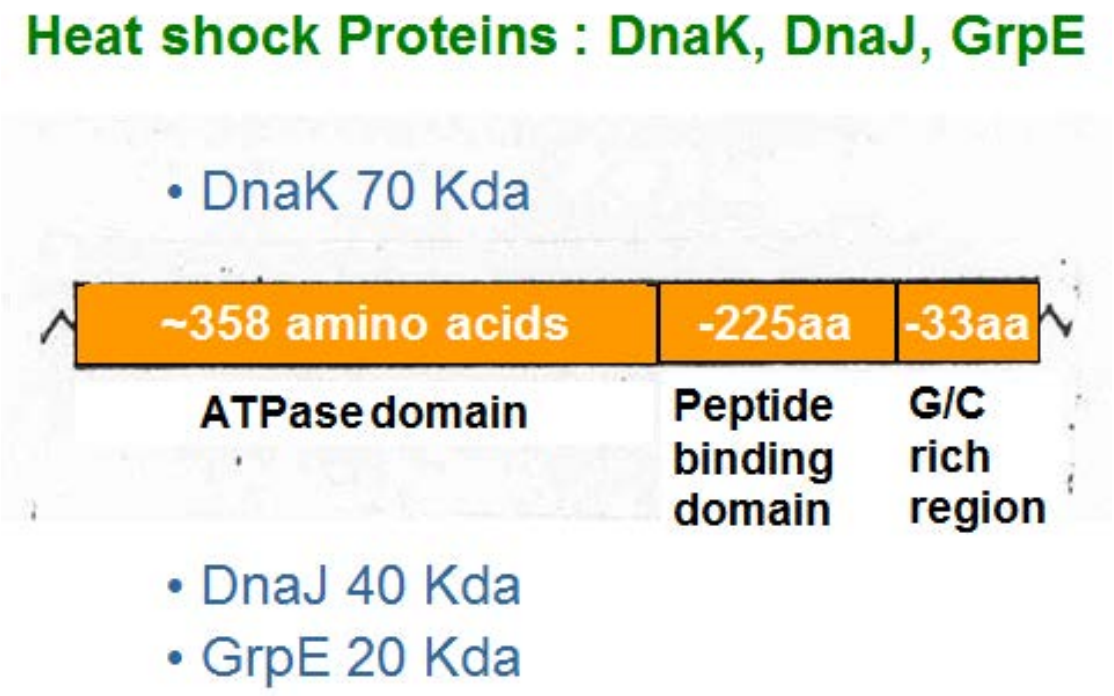


Figure 2.7 Approximate sizes of DnaK, DnaJ, and GrpE (Minder et al., 1997)

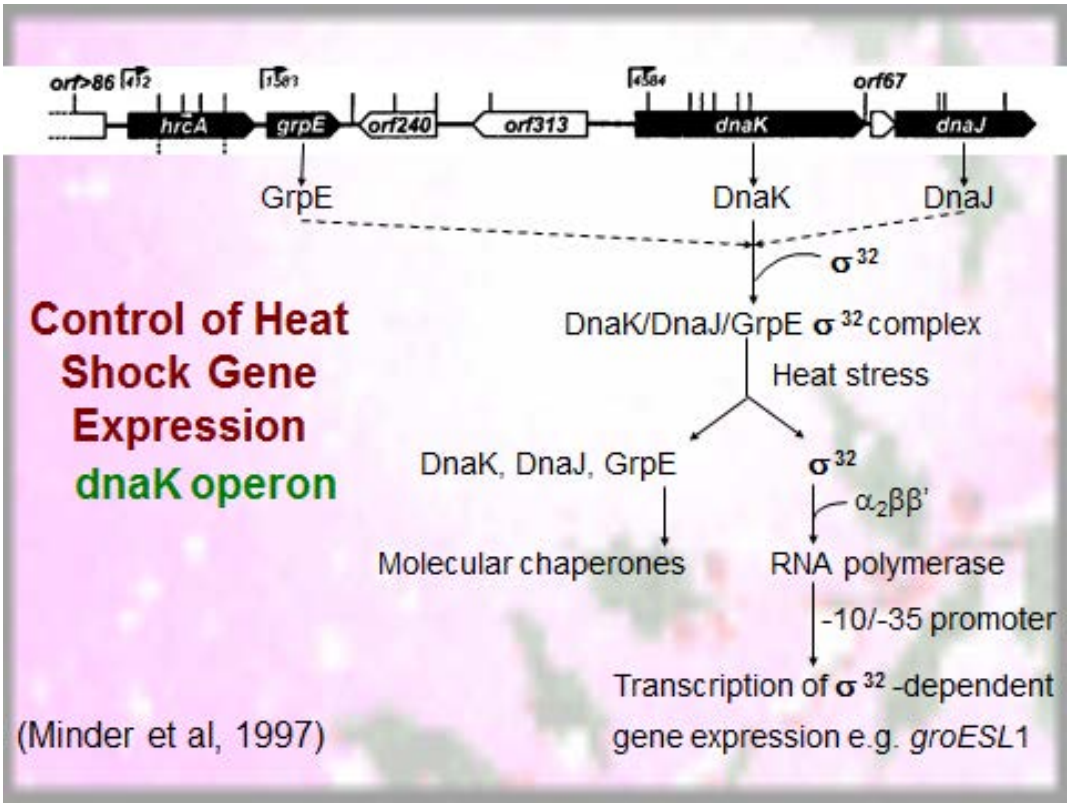


Figure 2.8 Function of DnaK in the control of gene expression under heat shock condition in *Bradyrhizobium* spp.

2.2 Molecular diversity of soybean rhizobia in other countries

Molecular characterization of soybean rhizobia in other countries consists of PCR-RFLP of several genes and spacer regions. For example, Chen et al.(2004) reported the characterization of 25 strains of soybean rhizobia from Shennongjia forest reserve, People's Republic of China, where soils from collection sites of different altitudes (500m, 1060m, 1500m, 1950m, 2400m, and 3100m) were acidic with pHs ranging from 4.6 to 5.6. No soybean rhizobia were collected from 500m and 1060m sampling sites. All isolated strains were found to be fast-growing soybean rhizobium *Sinorhizobium fredii* with mean generation time between 2.0 h to 3.4 h.

In 2008, Vinuesa et al. used MLSA to analyse 33 reference strains and 76 rhizobial strains isolated from root nodules of soybean grown in soil samples from Myanmar, India, Nepal, and Vietnam. The phylogenetic tree constructed with concatenated sequences of *atpD-glnII-recA-rhoB* showed 15 Myanmar strains were in the same cluster as *B. elkanii,* 18 strains isolated from soil samples from Nepal were found in the same cluster as *B. japonicum* strainla, one Myanmar strain was found to be a novel lineage, 9 strains from soil samples from Vietnam and 4 strains isolated from soil samples from Myanmar formed the same cluster as *B. liaoningense.* Finally, 6, 21, and 2 strains isolated from soil samples from Myanmar, India, and Vietnam, respectively, were found in the same cluster as *B. yuanmingense.* Most of the phylogenetic relationships were supported by high bootstrap numbers between 0.8-1.0.

In 2009, Binde et al. used nucleotide sequences of 16S rDNAs to identify 54 strains of rhizobia including soybean rhizobia *Bradyrhizobium elkanii*, *B. japonicum*, *B. liaoningense*, and *B. yuanmingense* . Construction of a phylogenetic tree using the 16S rDNA sequences revealed *B. elkanii* , *B. japonicum*, *B. liaoningense*, and *B. yuanmingense* indicating close genetic relationships amongst these four soybean rhizobial species.

Multilocus Sequence Analysis had also been used to delineate species in other microsymbionts of legumes other than soybean rhizobia. In 2009, Rivas et al., employed Multilocus Sequence Analysis to determine if concatenated sequences of five house-keeping genes, namely, *atpD-recA-gyrB-rpoB-dnaK* could be used to delineate species for 16 newly-isolated strains from leguminous plants *Lupinus albus, Arachis hypogaea,* and *Ornithopus compressus* from Spain. Primers were designed to amplify each gene from 45 strains which consisted of reference strains representing

named species and the 16 isolated strains. Phylogenetic trees obtained from partial sequences of each gene and from concatenated sequences as shown in Figure 2.9 did not group *Bradyrhizobium* spp. MCLA07, MCLA12, MCLA22 and MCLA23 isolated from *Lupinus albus* from Salamanca, Spain, into the same cluster as the 4 *Bradyrhizobium* spp. RLA08, RLA09, RLA10, and RLA11 which were isolated from *L. albus* from León, Spain. However, the following 8 *Bradyrhizobium* strains were grouped into separate clusters : 4 *Bradyrhizobium* strains MCAH03, MCAH06, MCAH12, and MCAH13 isolated from *Arachis hypogaea* in Salamanca, Spain, and 4 *Bradyrhizobium* strains MCOC04, MCOC05, MCOC23, and MCOC24 isolated from the host plant *Ornithopus compressus* in Salamanca, Spain. From the phylogenetic tree constructed from the concatenated sequences, the 12 *Bradyrhizobium* strains isolated from Salamanca, Spain, were identified as *Bradyrhizobium canariense*, while the other 4 *Bradyrhizobium* strains RLA08, RLA09, RLA10, and RLA11 which were isolated from *L. albus* from León, Spain, were found to be closely related to *B. japonicum.* The results seemed to indicate Mutilocus Sequence Analysis could not yet be used in place of DNA-DNA hybridization to delineate the 16 newly-isolated *Bradyrhizobium* species. However, Rivas et al. (2009) suggested that with more sequencing data and the future inclusion of more reference strains, MLSA could eventually be used to delineate species.

Figure 2.9 Phylogenetic tree, calculated using the maximum likelihood method, based on the concatenated sequence data for the genes *atpD, recA, gyrB, rpoB* and *dnaK* (Rivas et al., 2009).

CHAPTER III
MATERIALS AND METHODS

3.1 Bacterial strains

150 bacterial strains in YM slant culture which were previously isolated in Laboratory 404, Tab Building, Chulalongkorn University, from root nodules of soybean (*Glycine max* L.) CV. Chiangmai2 grown in a 15X24 sq.m. experimental plot in Nongkula district, Phitsanulok province in August 2009 were used in this study.

3.2 RAPD-PCR DNA fingerprinting of bacterial isolates from Nongkula subdistrict

One loop of each root nodule bacterial isolate was spread onto an agar plate containing yeast extract mannitol medium (YM) with 0.25 $\mu l.ml^{-1}$ final concentration of congo red. (mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, yeast extract 0.5 g, deionized water 1 liter). Plates were incubated at 30°C. If colonies were observed after 1-day incubation, the isolates were reported as fast-growers. On the other hand, if colonies were observed after 5-day incubation, the isolates were regarded as slow-growers. RAPD-PCR DNA fingerprints of slow-growing isolates were obtained as follows : One loop of each slow-growing root nodule bacterial isolate was inoculated into 50 ml of YM in a 250 ml Erlenmeyer flask. Cells grown at 30 $^{\circ}C$, 200 rpm, for 4 days were harvested by centrifugation at 8000 rpm, 4 $^{\circ}C$, 5 min, washed once with 0.85% NaCl to get rid of polysaccharides. Cells were broken by incubation for 1 h with lysozyme in 100 µl saline-EDTA (2.5 $mg.ml^{-1}$), 400 µl TE buffer, 20 µl 10% SDS followed by freezing and thawing at –20$^{\circ}C$, 5 min and 80$^{\circ}C$, 5 min, twice. RNA was hydrolyzed by adding 250 µl of DNAzol^{TM} (Molecular Research Center). DNA was precipitated with 30 µl 3M sodium acetate and 500 µl ice-cold absolute ethanol with incubation at –80$^{\circ}C$ for 15 min., washed with 70% ethanol, air dried, and dissolved in sterilized distilled water overnight. Quantity and quality of chromosomal DNA preparation were determined by OD260, OD260/OD280 and 1.25% agarose gel electrophoresis (Sambrook et al., 1989).

DNA fingerprints of each root nodule bacterial isolate were obtained by RAPD-PCR using either RPO1 (Richardson et al., 1995) or CRL-7 (Mathis and McMillin, 1996) as the primer. PCR mixture consisted of 10 µl 2X *Taq* Master Mix (1.25 unit *Taq* DNA Polymerase, 1X ViBuffer A, 0.2mM dNTPs and 1.5mM $MgCl_2$), 0.5 µl (100 pmole. μl^{-1})

primer RPO1 or 0.5 µl (100 pmole. µl^{-1}) primer CRL-7, DNA 200 ng, and sterilized distilled water to 20 µl. PCR program was 95 °C 15 seconds, 55 °C 30 seconds, 72 °C 90 seconds for 5 cycles, 95 °C 15 seconds, 60 °C 30 seconds, 72 °C 90 seconds for 25 cycles, followed by 72 °C 10 minutes. PCR products were separated by 1.25% agarose gel electrophoresis (Sambrook et al., 1989), stained in 0.5 µg/ml Ethidium bromide and photographed under UV light on Bio-rad UV transilluminator equipped with Polaroid camera using FUJI 3000 B Polaroid film.

3.3 Grouping of isolates and dendrogram construction from DNA fingerprints

Root nodule isolates with identical RAPD-PCR DNA fingerprints using either RPO1 or CRL-7 as the primer were assigned to the same strains. Dendrograms of RPO1-DNA fingerprints and CRL-7- DNA fingerprints of the isolated strains as well as some soybean rhizobial STB strains as reported by Maruekarajtinplaeng, (2010) were constructed with DNA Fingerprinting II Informatix software version 3.0 provided by the Bio-Rad Laboratories (Thailand) Co., Ltd.

3.4 Selection and authentication of bacterial strains

Five strains were selected from the dendrogram constructed from the DNA fingerprints. Each strain was grown in YM broth for 4 days as described in section 3.1. Five ml of each bacterial suspension were added onto germinating seeds (*Glycine max* cv Chiangmai 60) in Leonard jars as described by Somasegaran and Hoben (1994). Leonard jars were placed in a randomized complete block design experiment in a 28 °C - 32 °C temperature-controlled greenhouse for 28 days before the observation of root nodules when soybean plants were at R4 stage with 50% of soybean plants had at least one flower (www.natres.psu.ac.th/Department/PlantScience). If root nodules were observed, the bacterial strains were determined to be soybean rhizobia. On the contrary, if root nodules were not observed, the bacterial strains were not soybean rhizobia. Total nitrogen of the whole soybean plant as grown in Leonard jars as described by Somasegaren and Hoben (1994) was determined by the Kjeldahl method using the service of the Food Testing Center of Chulalongkorn University. At least 5 strains of soybean rhizobia which yielded relatively high total nitrogen content and large numbers of crown nodules with pink tissue were selected for Multilocus Sequence Analysis.

3.5 Flagella staining

One loop of each selected soybean rhizobial strain was grown in 3 ml of YM broth at room temperature for 48 h. Sample was dropped onto a copper grid of a

Transmission Electron Microscope. Cells were stained with 0.1% Phosphotungstic acid and dried overnight before observing under the Transmission Electron Microscope at the Research Technology and Equipment Center of Chulalongkorn University.

3.6 Multilocus Sequence Analysis in selected soybean rhizobia

Partial nucleotide sequences of 5 genes, namely, 16S rDNA, *dnaK*, *glnII*, *nifH* and *recA* were obtained for 5 soybean rhizobium strains isolated from Nongkula subdistrict, Phitsanulok province. All the selected 5 soybean rhizobium strains had different DNA fingerprints. Primers 27f and 1492r for the amplification of 16S rDNA were as described by Dorsch and Stackerbrandt (1992). Primers for the amplification of partial *nifH* were as described by Siras Chulanpakorn (2007). Primers for the amplification of partial fragments of the remaining genes were designed by downloading sequences of the genes from GenBank to do multiple alignments and conserved sequences were used as forward and reverse primers as shown in Appendix D.

Composition of PCR mixture and PCR program for the amplification of 16S rDNA were as follows: 10 µl 2X *Taq* Master Mix (1.25 unit *Taq* DNA Polymerase, 1X ViBuffer A, 0.2mM dNTPs and 1.5mM $MgCl_2$), 0.5 µl (100 pmole. μl^{-1}) primer 27f and 0.5 µl (100 pmole. μl^{-1}) primer 1492r, DNA 200 ng, and sterilized distilled water to 20 µl. PCR program was as follows: 95^{o}C 30 minutes, 48^{o}C 1 minute, 72^{o}C 2 minutes (30 cycles) followed by 48^{o}C 1 minute, 72^{o}C 5 minutes (1 cycle).

Sequences of the primers 27f and 1492r were as described by Dorsch and Stackebrandt (1992) : 27f (9-27)* : 5'GAGTTTGATCCTGGCTCAG3', 1492r (1492-1512)* : 5'ACGGCTACCTTG TTACGACCT3'

* Positions of nucleotides on consensus sequence of 16S rDNA of *E. coli*

Composition of PCR mixture and PCR program for amplification of partial fragments of each of the other four genes were as described for RAPD-PCR fingerprinting in section 3.1

PCR products were sent to the Faculty of Medicine, Ramathibodi Hospital Research Center, for sequencing by thermal cycler (Applied Biosystem 2002, using BigDye® Terminator V3.1), Cycle Sequencing protocol and DNA Sequencer using DNA Sequencer ABI 3100 Genetic analyzer. Nine primer (27f,1241f, 1492r, 1385r, 1110r,

907r, 787r, 509r, and 343r) as described by Dorsch and Strakerbrardt (1992) were used as sequencing primers for 16S rDNA. Each set of forward and reverse primers for the amplification of the other forward genes were also used as the sequencing primers. All primers were synthesized by Macrogen (Korea). In addition, nucleotide sequences of the five genes of reference strains deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) as shown in Table 3.1 were used in Multilocus Sequence Analysis (MLSA) with freeware programs including BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Construction of phylogenetic trees were obtained by using program MEGA5.2 (http://www.megasoftware.net/mega.php).

Table 3.1 GenBank accession numbers for the sequences downloaded used in MLSA in this study**.**

Strain	16S rDNA	*dnaK*	*glnII*	*recA*	*nifH*
BGA-1	AJ558024	FJ970202	AY386772	AY591558	AY386784
CB15	AJ227757	AE005673	AE005673	AE005673	
CCBAU 10071			AY386780	AY591566	EU818927
CCBAU 23283	HM107163		HM107247	HM107229	HM107279
CCBAU 25551	HQ231447		HQ231623	HQ231579	HQ231535
CCBAU 45291	HM107158		HM107242	HM107224	HM107274
CCBAU 45394	HM107164	KC508989	HM107248	HM107230	HM107280
DSM 19922					GU256451
SEMIA 511	FJ390901	FJ390982	FJ391022	FJ391142	HQ259527
SEMIA 587	AF234890	FJ390985	FJ391025	FJ391145	HQ259549
SEMIA 5011	FJ390893	FJ390989	FJ391029	FJ391149	HQ259551
SEMIA 5025	FJ390935	FJ390991	FJ391031	FJ391151	HQ259552
SEMIA 5026	FJ390894	FJ390992	FJ391032	FJ391152	HQ259532
SEMIA 5045	FJ390924	FJ390994	FJ391034	FJ391154	HQ259533
SEMIA 5062	FJ390900	FJ390995	FJ391035	FJ391155	HQ259554
SEMIA 5079	AF234888	FJ390996	FJ391036	FJ391156	HQ259534
SEMIA 5080	AF234889				
SEMIA 6319	AY904774	FJ391018	FJ391058	FJ391178	HQ259545
SR69		EU818928	EU818932	EU818936	
SR135		FJ514049	FJ514061	FJ514055	FJ514070
USDA76	HQ233240	AY328392	AY599117	AY591568	
USDA94	AF363152	AY328393	AY599118		AY599092
USDA110	BA000040	BA000040	BA000040	BA000040	BA000040

CHAPTER IV
RESULTS

4.1 RAPD-PCR fingerprinting of bacterial isolates from Nongkula subdistrict

Table 4.1 showed codes of 150 bacterial isolates obtained from root nodules of soybean cv. Chiangmai 2 grown in a 15 x 24 sq. m. experimental plot in Nongkula subdistrict, Phitsanulok province. 116 isolates which were found to be slow-growers were used in RAPD-PCR fingerprinting using either RPO1 or CRL-7 as the primer. All the fingerprints are shown in Figures 4.1-4.7.

Table 4.1 Determination of fast- or slow-growing property of bacteria isolated from root nodules of soybean cv. Chiangmai 2 grown in an experimental plot in Nongkula subdistrict, Phitsanulok province in August 2009.

Code of bacterial isolates	Fast(F) or slow(S) growers	Code of bacterial isolates	Fast(F) or slow(S) growers	Code of bacterial isolates	Fast(F) or slow(S) growers
NKL09064	F	NKL09192	S	NKL09232	S
NKL09065	F	NKL09194	S	NKL09233	S
NKL09066	F	NKL09195	F	NKL09237	S
NKL09073	F	NKL09196	S	NKL09239	S
NKL09074	F	NKL09197	F	NKL09240	S
NKL09083	F	NKL09203	S	NKL09243	S
NKL09096	F	NKL09207	S	NKL09244	F
NKL09106	F	NKL09210	S	NKL09246	S
NKL09107	F	NKL09212	S	NKL09248	S
NKL09110	F	NKL09213	S	NKL09250	S
NKL09112	F	NKL09215	F	NKL09251	S
NKL09113	S	NKL09216	S	NKL09252	S
NKL09114	F	NKL09217	S	NKL09253	S
NKL09115	F	NKL09219	S	NKL09255	S
NKL09116	F	NKL09220	F	NKL09256	S
NKL09114	F	NKL09225	S	NKL09257	S
NKL09119	S	NKL09226	S	NKL09259	S
NKL09125	F	NKL09229	S	NKL09260	S
NKL09126	F	NKL09231	S	NKL09262	S
NKL09264	F	NKL09677	S	NKL091011	S
NKL09266	S	NKL09679	S	NKL091012	S
NKL09269	S	NKL09683	S	NKL091013	S

Code of bacterial isolates	Fast(F) or slow(S) growers	Code of bacterial isolates	Fast(F) or slow(S) growers	Code of bacterial isolates	Fast(F) or slow(S) growers
NKL09270	S	NKL09686	S	NKL091017	S
NKL09271	S	NKL09689	S	NKL091018	S
NKL09272	S	NKL09690	S	NKL091019	S
NKL09273	S	NKL09691	S	NKL091020	S
NKL09276	S	NKL09692	S	NKL091021	S
NKL09278	F	NKL09693	S	NKL091022	S
NKL09279	S	NKL09694	S	NKL091023	S
NKL09280	S	NKL09699	S	NKL091024	S
NKL09282	S	NKL09701	S	NKL091044	S
NKL09283	S	NKL09703	S	NKL091045	S
NKL09284	S	NKL09706	S	NKL091046	S
NKL09288	S	NKL09707	S	NKL091047	S
NKL09652	S	NKL09812	F	NKL091048	S
NKL09653	S	NKL09813	S	NKL091049	S
NKL09659	S	NKL09816	S	NKL091050	S
NKL09660	F	NKL09818	S	NKL091051	S
NKL09662	S	NKL09819	S	NKL091052	S
NKL09666	S	NKL09820	F	NKL091053	S
NKL09667	S	NKL09821	F	NKL091054	S
NKL09668	S	NKL09822	F	NKL091055	S
NKL09669	S	NKL09823	F	NKL091056	S
NKL09670	S	NKL09824	S	NKL091057	S
NKL09671	S	NKL091005	F	NKL091091	S
NKL09672	S	NKL091007	F	NKL091095	S
NKL09674	S	NKL091008	S	NKL091096	F
NKL09675	S	NKL091009	S	NKL091099	F
NKL09676	S	NKL091010	S	NKL091101	S
NKL091103	S	NKL091106	S	NKL091136	S

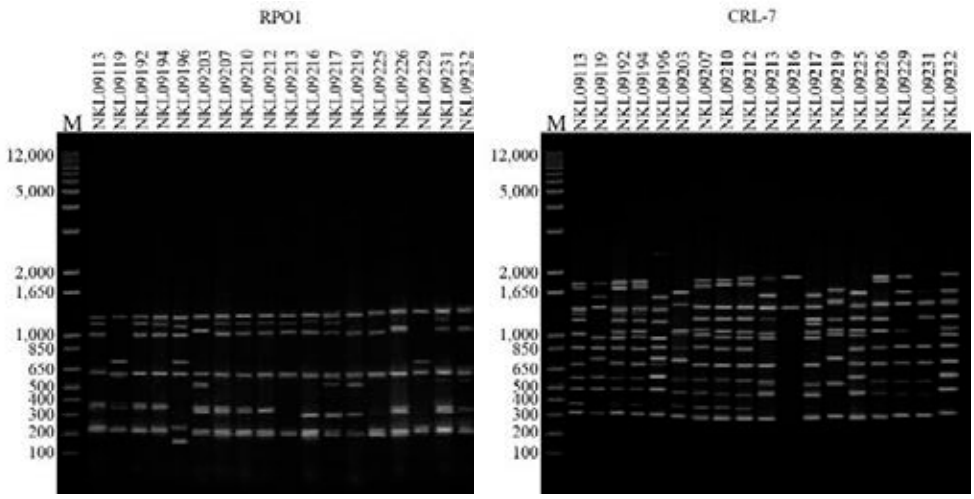


Figure 4.1 RAPD-PCR fingerprints of slow-growing bacterial isolates obtained from root nodules of soybean cv. Chiangmai 2 grown in an experimental plot in Nongkula subdistrict, Phitsanulok province in August 2009. Identical fingerprints showed the following isolates were the same strains NKL09119=NKL09229, NKL09192=NKL09194 = NKL09207= NKL09210= NKL09212.

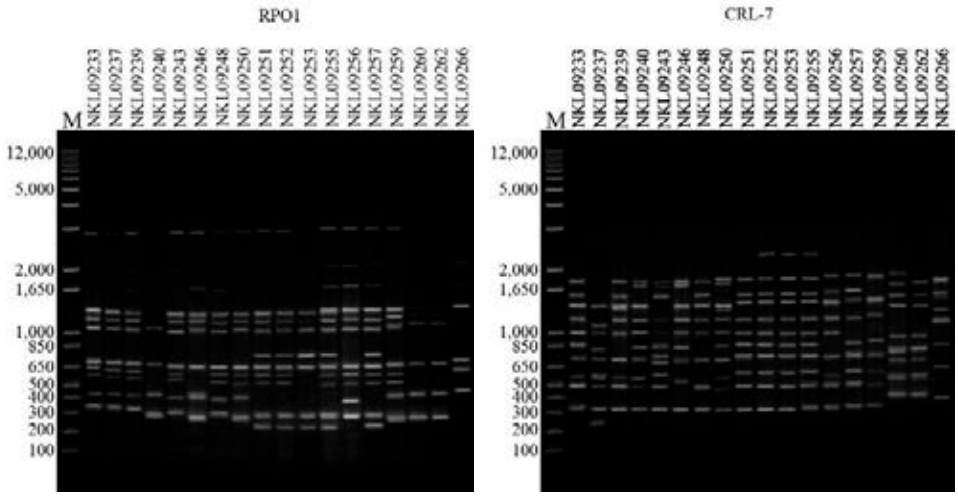


Figure 4.2 RAPD-PCR fingerprints of slow-growing bacterial isolates obtained from root nodules of soybean cv. Chiangmai 2 grown in an experimental plot in Nongkula subdistrict, Phitsanulok province, in August 2009. Identical fingerprints showed the following isolates were the same strains: NKL09233=NKL09251=NKL09257, NKL09239=NKL09248, NKL09240=NKL09246= NKL09250, NKL09252=NKL09253= NKL09255, NKL09260= NKL09262.

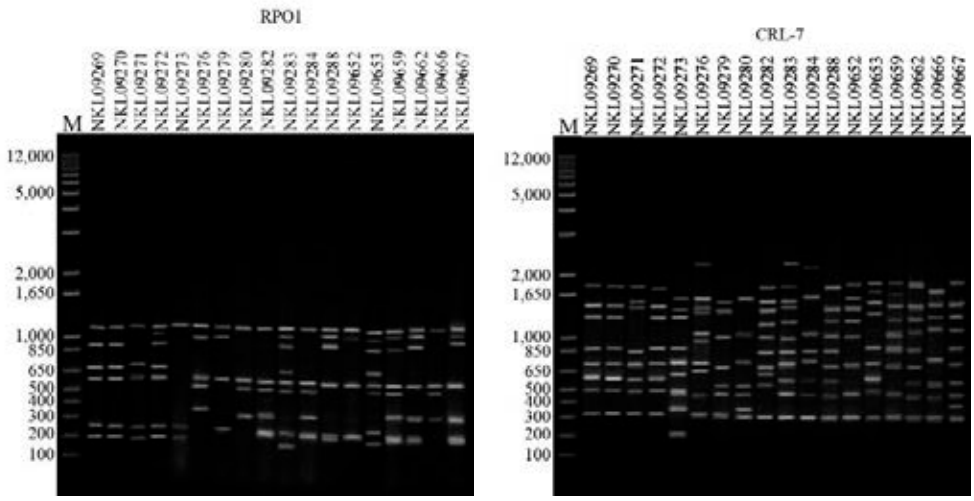


Figure 4.3 RAPD-PCR fingerprints of slow-growing bacterial isolates obtained from root nodules of soybean cv. Chiangmai 2 grown in an experimental plot in Nongkula subdistrict, Phitsanulok province, in August 2009. Identical fingerprints showed the following isolates were the same strains: NKL09269=NKL09270=NKL09272, NKL09288=NKL09662.

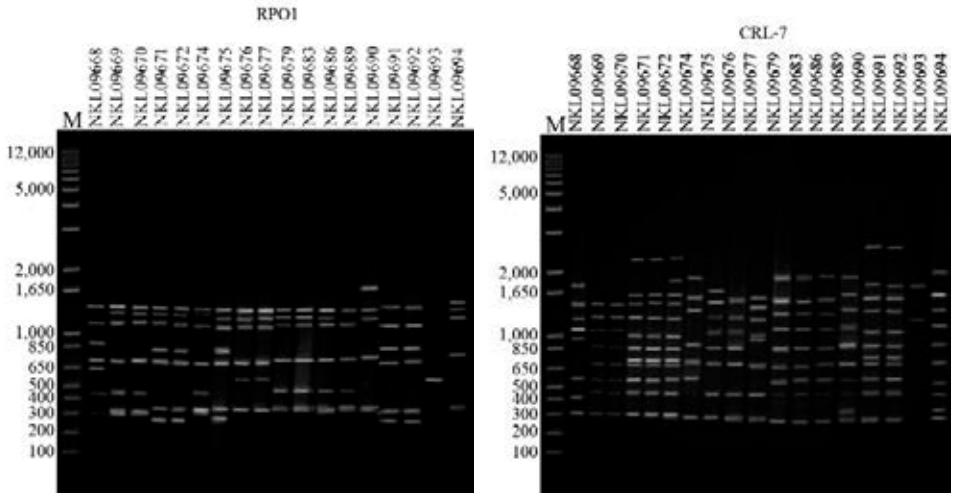


Figure 4.4 RAPD-PCR fingerprints of slow-growing bacterial isolates obtained from root nodules of soybean cv. Chiangmai 2 grown in an experimental plot in Nongkula subdistrict, Phitsanulok province, in August 2009. Identical fingerprints showed the following isolates were the same strains: NKL09669=NKL09670, NKL09671=NKL09672 =NKL09674=NKL09691=NKL09692, NKL09677=NKL09679, NKL09683=NKL09686= NKL09689=NKL09690= NKL09694.

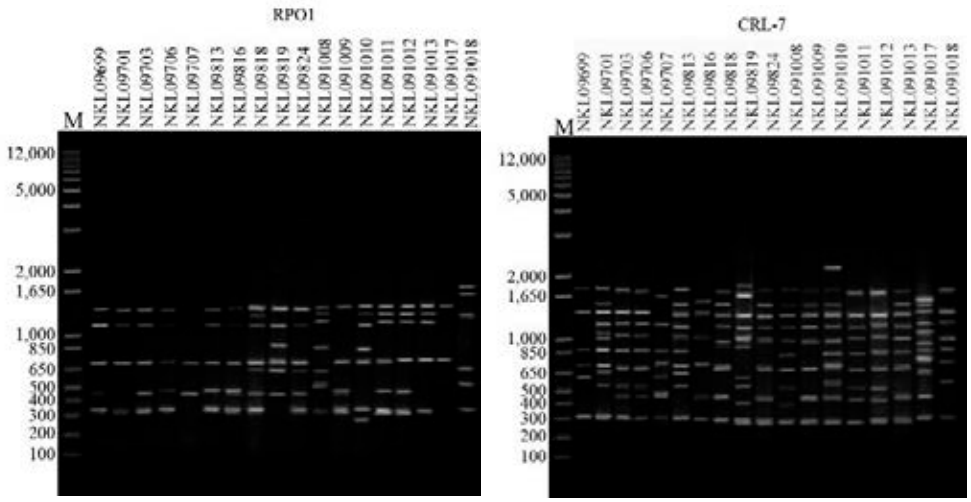


Figure 4.5 RAPD-PCR fingerprints of slow-growing bacterial isolates obtained from root nodules of soybean cv. Chiangmai 2 grown in an experimental plot in Nongkula subdistrict, Phitsanulok province, in August 2009. Identical fingerprints showed the following isolates were the same strains: NKL09701=NKL09813, NKL09703= NKL09706=NKL091011=NKL091012, NKL09818=NKL09824= NKL091009= NKL091023, NKL091010=NKL091013.

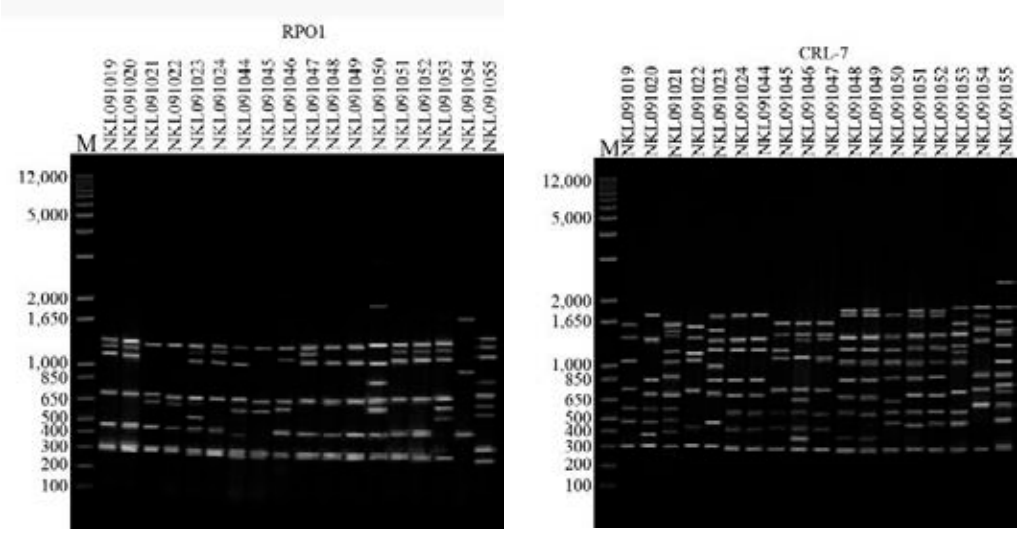


Figure 4.6 RAPD-PCR fingerprints of slow-growing bacterial isolates obtained from root nodules of soybean cv. Chiangmai 2 grown in an experimental plot in Nongkula subdistrict, Phitsanulok province , in August 2009. Identical fingerprints showed the following isolates were the same strains : NKL091022=NKL091045, NKL091024= NKL091044, NKL091046=NKL091047, NKL091048= NKL091049, NKL091051= NKL091052.

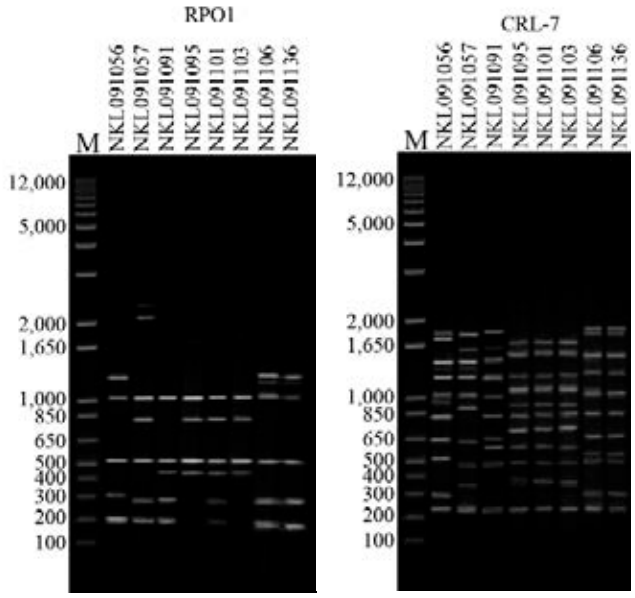


Figure 4.7 RAPD-PCR fingerprints of slow-growing bacterial isolates obtained from root nodules of soybean cv. Chiangmai 2 grown in an experimental plot in Nongkula subdistrict, Phitsanulok province, in August 2009. Identical fingerprints showed the following isolates were the same strains :NKL091056=NKL091106=NKL091136 , NKL091057=NKL091091, NKL091095= NKL091101= NKL091103.

Since RAPD-PCR fingerprints of some isolates which were shown in different gels were identical, these isolates were also the same strains. Table 4.2 summarized all the 43 isolates that were found to be 43 strains.

Table 4.2 Summary of all the slow-growing bacterial isolates obtained from the experimental plot in Nongkula subdistrict, Phitsanulok province, that were the same strains.

Strains	Isolates	Strains	Isolates	Strains	Isolates
NKL09113	NKL09113		NKL09818		NKL09676
	NKL091048		NKL09824		NKL091054
	NKL091049		NKL091009	NKL09243	NKL09243
NKL09119	NKL09119		NKL091023	NKL09252	NKL09252
	NKL09229	NKL09225	NKL09225		NKL09253
NKL09192	NKL09192		NKL091008		NKL09255
	NKL09194		NKL001053		NKL09283
	NKL09207	NKL09226	NKL09226	NKL09259	NKL09259
	NKL09210		NKL09240		NKL09269
	NKL09212		NKL09246		NKL09270
	NKL091056		NKL09250		NKL09272
	NKL091106		NKL091051		NKL091024
	NKL091136		NKL091052		NKL091044
NKL09196	NKL09196		NKL091057	NKL09260	NKL09260
	NKL09671		NKL091091		NKL09262
	NKL09672	NKL09231	NKL09231		NKL09653
	NKL09674		NKL091019	NKL09266	NKL09266
	NKL09691	NKL09232	NKL09232	NKL09271	NKL09271
	NKL09692		NKL09701	NKL09273	NKL09273
NKL09203	NKL09203		NKL09813	NKL09276	NKL09276
	NKL091020	NKL09233	NKL09233	NKL09279	NKL09279
NKL09213	NKL09213		NKL09251		NKL09816
	NKL09256		NKL09257	NKL09280	NKL09280
	NKL09703		NKL091010	NKL09282	NKL09282
	NKL09706		NKL091013	NKL09284	NKL09284
	NKL091011		NKL091055	NKL09288	NKL09288
	NKL091012	NKL09237	NKL09237		NKL09662
NKL09216	NKL09216	NKL09239	NKL09239		NKL091050
NKL09217	NKL09217		NKL09248	NKL09659	NKL09659
NKL09219	NKL09219		NKL09652		NKL091018
NKL09666	NKL09666		NKL09670		NKL091021

Strains	Isolates	Strains	Isolates	Strains	Isolates
NKL09667	NKL09667	NKL09675	NKL09675	NKL091022	NKL091022
	NKL09683		NKL09699		NKL091045
	NKL09686	NKL09677	NKL09677	NKL091046	NKL091046
	NKL09689		NKL09679		NKL001047
	NKL09690	NKL09693	NKL09693	NKL091095	NKL091095
	NKL09694	NKL09707	NKL09707		NKL091101
NKL09668	NKL09668	NKL09819	NKL09819		NKL091103
NKL09669	NKL09669	NKL091017	NKL091017		

4.2 Authentication of bacterial strains

All the 43 slow-growing strains were authenticated to find out if they were soybean rhizobia. Authentication results of all the 43 slow-growing strains were shown in Appendix E. Results of total N of the whole plant (g/100 g plant) of all the soybean plants inoculated with each of the 43 strains were shown in Appendix E.

4.3 Dendrogram construction from RAPD-PCR fingerprints

RAPD-PCR fingerpints using either RPO1 or CRL-7 as the primer for the 43 slow-growing soybean rhizobial strains were used to construct two dendrograms as shown in Figures 4.8 and 4.9.

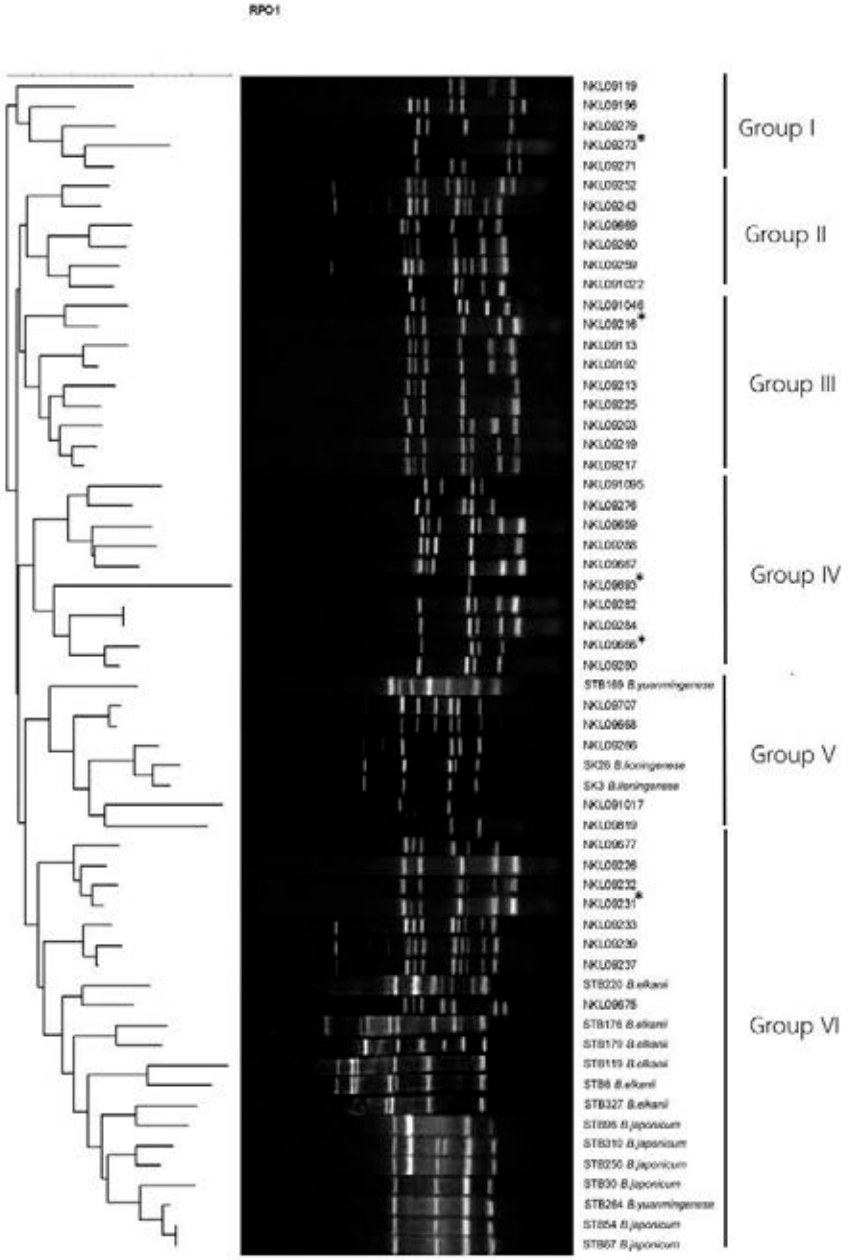


Figure 4.8 Dendrogram constructed with RPO1 RAPD-PCR fingerprints of the 43 slow-growing soybean shizobial strains isolated from an experimented plot in Nongkula subdistrict, Phitsanulok province, using DNA Fingerprinting II Informatix software version 3.0 provided by the Bio-Rad Laboratories (Thailand) Co., Ltd. Some reference STB soybean rhizobia strains (Maruekarajtinplaeng, 2010) were also used in the construction of the dendrogram. (* strains selected for MLSA).

The dendrogram constructed with RPO1-RAPD-PCR fingerprints as shown in Figure 4.8 showed six distinct groups as follows :

Group I : NKL09119, NKL09196, NKL09279, NKL09273, NKL09271

Group II : NKL09252, NKL09243, NKL09669, NKL09260, NKL09259, NKL091022

Group III : NKL091046, NKL09216, NKL09113, NKL09192, NKL09213, NKL09225, NKL09203, NKL09219, NKL09217

Group IV : NKL091095, NKL09276, NKL09659, NKL09288, NKL09667, NKL09693, NKL09282, NKL09284, NKL09666, NKL09280

Group V : *B.yuanmingense* STB169, NKL09707, NKL09668, NKL09266, *B.liaoningense* SK26, *B.liaoningense* SK3, NKL091017, NKL09819

Group VI : NKL09677, NKL09226, NKL09232, NKL09231, NKL09233, NKL09239, NKL09237, *B.elkanii* STB220, NKL09675, *B.elkanii* STB176, *B.elkanii* STB179, *B.elkanii* STB119, *B.elkanii* STB8, *B.elkanii* STB327, *B.japonicum* STB96, *B.japonicum* STB310, *B.japonicum* STB250, *B.japonicum* STB30, *B.yuanmingense* STB264, *B.japonicum* STB54, *B.japonicum* STB67.

Since most of the isolated soybean rhizobial strains were clustered in Groups I, II, III, and IV, it was relatively difficult to select five soybean rhizobial strains from the Groups to do MLSA. The dendrogram showed NKL09707 and NKL09668 had a close relationship with *B.yuanmingense* STB16 while NKL09266, NKL091017, NKL09819 had a close relationship with *B.liaoningense* strains SK26 and SK3. NKL09675 was found to have a close relationship with *B.elkanii* STB220. In addition all the reference strains that were either *B.elkanii* or *B.japonicum* were found to have a close relationship and were grouped in GroupVI while *B.yuanmingense* STB169 and STB264 were found to have a relatively distant relationship.

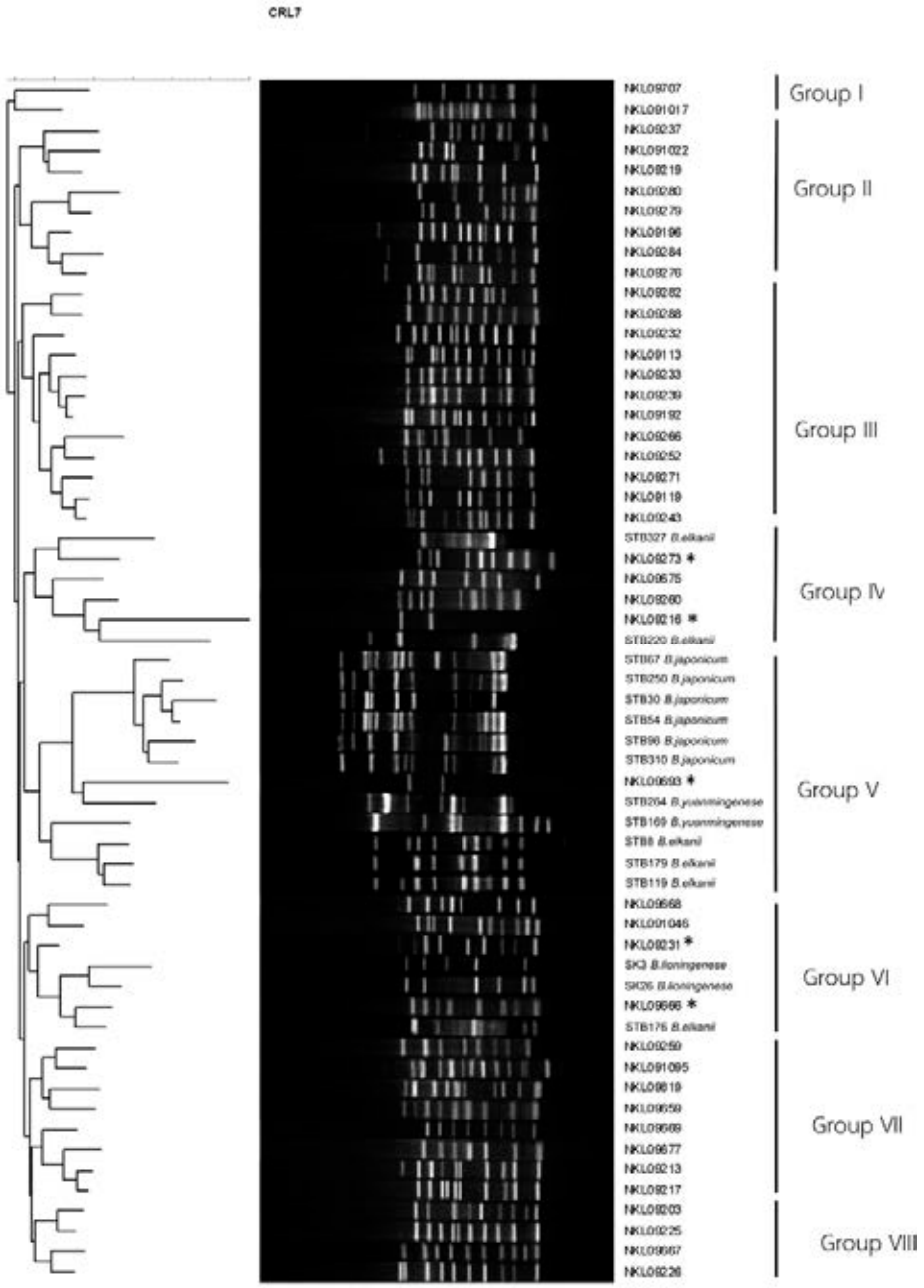


Figure 4.9 Dendrogram constructed from CRL-7 RAPD-PCR fingerprints of the 43 slow-growing soybean rhizobial strains obtained from an experiment plot in Nong kula subdistrict, Phitsanulok province. Some STB soybean rhizobial strains obtained from Maruekarajtinplaeng (2010) were used in the construction of the dendrogram using DNA Fingerprinting II Informatix software version 3.0 provided by the Bio-Rad Laboratories (Thailand) Co., Ltd. (* strains selected for MLSA).

The CRL-7 RAPD-PCR dendrogram as shown in Figure 4.9 showed the soybean rhizobial strains could be grouped into eight distinct groups as follows :

Group I : NKL09707, NKL091017

Group II : NKL09237, NKL091022, NKL09219, NKL09280, NKL09279, NKL09196, NKL09284, NKL09276

Group III : NKL09282, NKL09288, NKL09232, NKL09113, NKL09233, NKL09239, NKL09192, NKL09266, NKL09252, NKL09271, NKL09119, NKL09243

Group IV : *B.elkanii* STB327, NKL09273, NKL09675, NKL09260, NKL09216, *B.elkanii* STB220

Group V : *B.japonicum* STB67, *B.japonicum* STB250, *B.japonicum* STB30, *B.japonicum* STB54, *B.japonicum* STB96, *B.japonicum* STB310, NKL09693, *B.yuanmingense* STB264, *B.yuanmingense* STB169, *B.elkanii* STB8, *B.elkanii* STB179, *B.elkanii* STB119

Group VI : NKL09668, NKL091046, NKL09231, *B.liaoningense* SK3, *B.liaoningense* SK26, NKL09666, *B.elkanii* STB176

Group VII : NKL09259, NKL091095, NKL09819, NKL09659, NKL09669, NKL09677, NKL09213, NKL09217

Group VIII : NKL09203, NKL09225, NKL09667, NKL09226

The two *B.elkanii* STB327 and STB220 reference strains were found to be closely related in Group IV while *B.elkanii* reference strains STB179 and STB119 were found to be closely related in the same Group V with *B.japonicum* and *B.yuanmingense* strains while *B.elkanii* STB176 was found to be closely related to *B.liaoningense* reference strains SK3 and SK26 in Group VI. In addition, the CRL-7 RAPD-PCR fingerprints dendrogram showed a close relationship between *B.japonicum* and *B.yuanmingense* reference strains in Group V and a close relationship between *B.liaoningense* SK3 and SK26 in Group VI.

Five bacterial strains were selected for Multilocus Sequence Analysis (MLSA) based on the ability to identify strains from CRL-7 RAPD-PCR fingerprints. For example, NKL09273 was selected because the strain was found to have a close relationship with *B.elkanii* STB327*;* NKL09216 was chosen because of its close relationship with *B.*elkanii STB220; NKL09693, NKL09231, and NKL09666 were chosen because of their close relationships with *B.yuanmingense* STB264*, B.liaoningense* SK3 and *B.elkanii* STB176 respectively. In addition, it is interesting to note that NKL09273, NKL09216, and NKL09666 which were predicted to be *B.elkanii* by CRL-7 fingerprints were find to have relatively distant relationships. It was expected that MLSA would help explain the distant relationships.

4.4 Polyphasic taxonomy of the five selected soybean rhizobial strains

4.4.1 Colony morphology

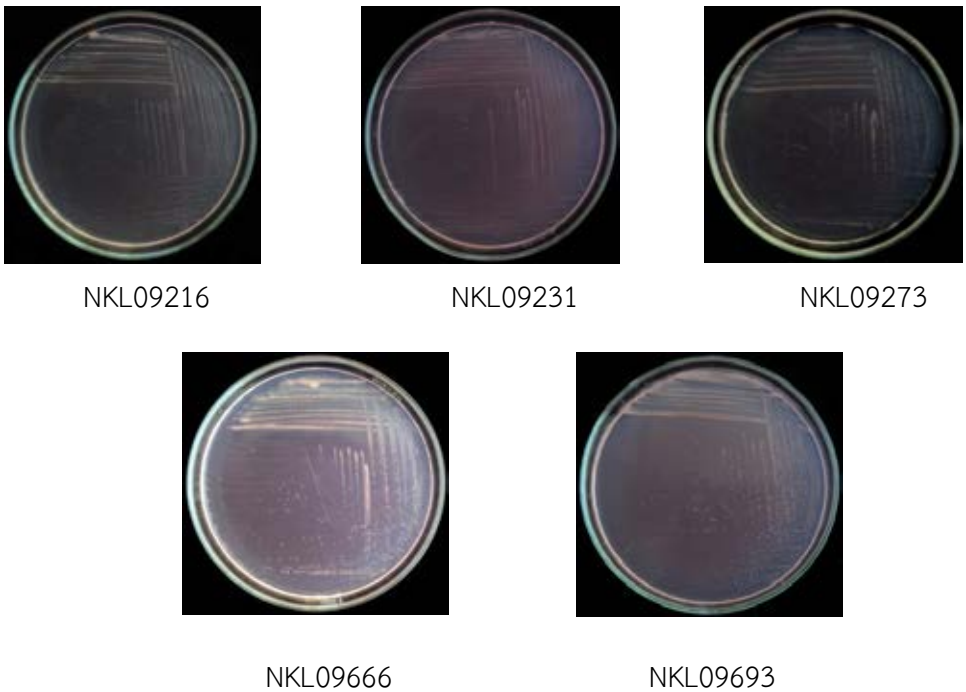


Figure 4.10 Colony morphology of the five selected soybean rhizobial strains on YMA with congo red incubated at 30°C for 5 days.

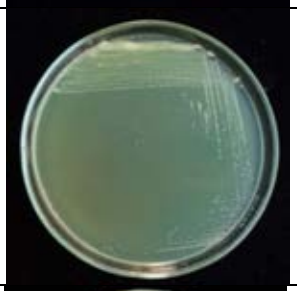
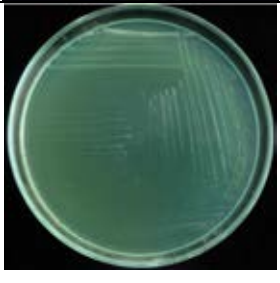
Figure 4.10 showed all the five selected soybean rhizobial strains were slow-growing with less than 0.1 mm colony diameter after 5-day incubation at 30°C. All colonies did not absorb congo red as expected (Somasegaran and Hoben, 1994).

Colony morphology of the rest of the 43 isolated strains was shown in Appendix F. Colony morphology was found to be similar in all the 43 isolated strains.

4.4.2 Bromothymol blue reactions

Table 4.3 showed bromothymol blue reactions of the five selected soybean rhizobial strains. All the five strains were found to secrete acidic products after 5- and 10- day incubation at 30°C. Strain NKL09273 showed on unusually high amount of polysaccharides after 10- day incubation. Bromothymol blue reactions of the rest of the strains revealed acidic products were secreted as shown in Appendix G. In addition, some other strains (NKL09271, NKL09659, NKL09819, and NKL091095) were found to secrete unusually high amounts of polysaccharides as observed in the selected strain NKL09273 after 10- day incubation at 30°C.

Table 4.3 Bromothymol blue reactions of the five selected soybean rhizobial strains.

Strains	days	
	5 days	10 days
NKL09216		
NKL09231		
NKL09273		
NKL09666		
NKL09693		

4.4.3 Authentication tests of the five selected soybean rhizobial strains

Table 4.4 showed the averages of total soybean plant dry weight when each of the five selected soybean rhizobial strains was used in the authentication test. Results of total soybean plant dry weight as determined by the Kjeldahl method when each of the 43 strains was inoculated into Leonard jars were given in Appendix E. The results showed the total soybean plant by weight ranged from 0.01-1.00 g/100g plant. The selected strains yielded total plant dry weight in relatively high range values.

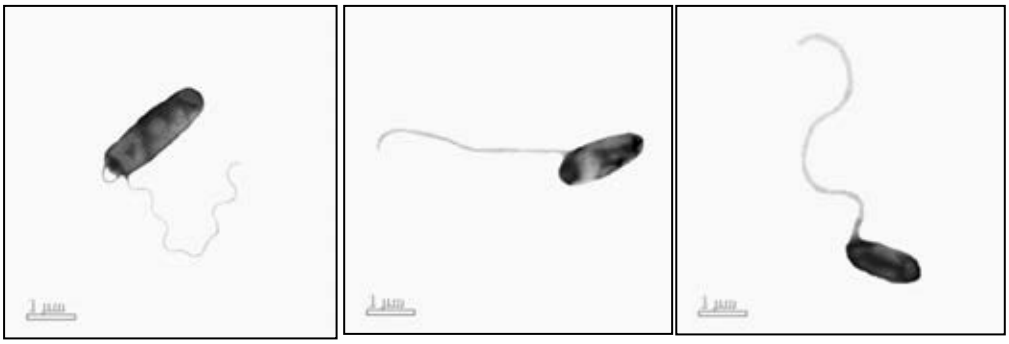
Table 4.4 Total nitrogen of the whole soybean plants as determined by the Kjeldahl method.

Total Nitrogen of the whole soybean plants as determined by the Kjeldahl method				
Determination	Strains	Total Nitrogen g/100g	Average	SD
1	NKL09216	0.67	0.605	0.092
2		0.54		
1	NKL09231	0.76	0.843	0.114
2		0.92		
1	NKL09273	0.61	0.685	0.106
2		0.76		
1	NKL09666	0.73	0.73	0
2		0.73		
1	NKL09693	0.89	0.84	0.071
2		0.79		

4.4.4 Negative staining for type and number of flagella

Figure 4.11 showed each of the five selected soybean rhizobial strains had one subpolar flagellum of different lengths.

NKL09216 NKL09231 NKL09273



NKL09666 NKL09693

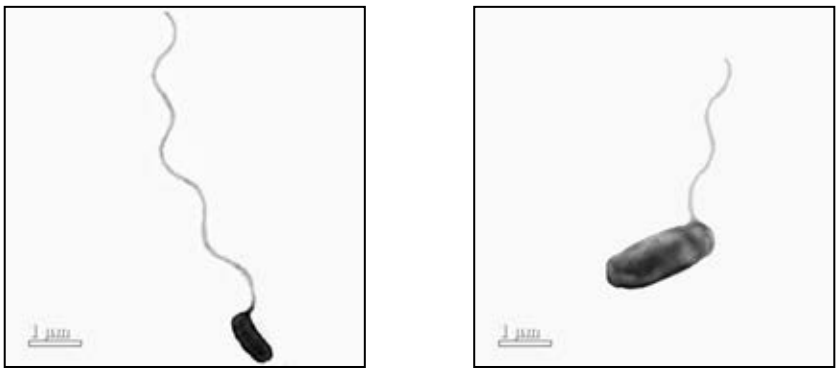


Figure 4.11 Type and number of flagella of the five selected soybean rhizobial strains as obtained by negative staining.

4.4.5 Multilocus Sequence Analysis in selected soybean rhizobia

In this thesis, identification of selected five strains of slow-growing soybean rhizobia was obtained by using partial sequences of genes 16S rDNA, *dnak, glnII, nifH* and *recA*.

4.4.5.1 Identification by 16S rDNA partial sequences

Figure 4.12 showed the isolated 16S rDNA of the five selected soybean rhizobial strains were approximately the same size of 1,500 bp.

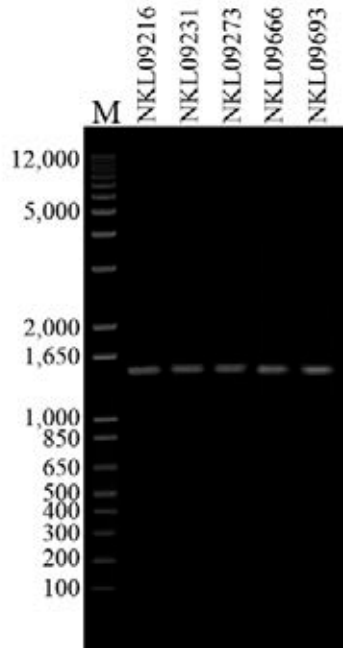


Figure 4.12 Isolated 16S rDNA of the five selected sobean rhizobial strains. All strains were found to contain 16S rDNA of approximately 1,500 bp

Table 4.5 showed identification of the 5 slow-growing soybean rhizobia NKL strains by using the Blast program to compare the obtained 16S rDNA sequences with those sequences in the GenBank database. The Blast program indicated the following results for the identification of the selected NKL strains using partial sequnces of 16S rDNA.

NKL09216 (determined length 1452 bp) could be related to *Bradyrhizobium yuanmingense* strain TTC4 or *Bradyrhizobium liaoningense* strain LYG2, All the compared sequences had the following homology: identities = 1447/1452 (99%), gap = 1/1452.

NKL09231 (determined length 1452 bp) could be related to *Bradyrhizobium yuanmingense* strain TTC4 or *Bradyrhizobium liaoningense* strain LYG2. All the compared sequences had the following homology: identities = 1447/1452(99%), gap = 1/1452.
NKL09273 (determined length 1339 bp) could be related to *Bradyrhizobium elkanii* strain STB179 or *Bradyrhizobium elkanii* strain STB120. All the compared sequences had the following homology: identities = 1339/1340(99%), gap = 1/1340.
NKL09666 (determined length 1451 bp) could be related to *Bradyrhizobium yuanmingense* strain TTC4 or *Bradyrhizobium liaoningense* strain LYG2. All the compared sequences had the following homology: with identities = 1450/1451(99%), gap = 0/1451.
NKL09693 (determined length 1451 bp) could be related to *Bradyrhizobium yuanmingense* strain TTC4 or *Bradyrhizobium liaoningense* strain LYG2. All the compared sequences had the following homology: identities = 1451/1451(100%), gap = 0/1451.

The number of nucleotides of 16S rDNA of NKL09273 was found to be 1339 bp which was much lower than these of the other four strains. The reason was because the PCR products obtained during sequencing using 27f or 1241f, or 1492r as the primer were contaminated as seen in the overlapping sequencing peaks for NKL09273 using one of the above-mentioned three primers as the sequencing primer as shown in Appendix H.

Table 4.5 Summary of the identification of the 5 slow-growing soybean rhizobia NKL strains based on partial 16S rDNA sequences.

Strain	Size of 16S rDNA product (bp)	Percent homology with sequences in GenBank	Identification
NKL09216	1452	1447/1452(99%) with 1 gap	*Bradyrhizobium yuanmingense* strain TTC4 or *B. liaoningense* strain LYG2
NKL09231	1452	1447/1452(99%) with 1 gap	*B.yuanmingense* strain TTC4 or *B. liaoningense* strain LYG2
NKL09273	1339	1406/1410(99%) with 3 gaps	*B. elkanii* strains STB179 or STB120
NKL09666	1451	1450/1451(99%) with no gap	*B.yuanmingense* strain TTC4 or *B. liaoningense* strain LYG2
NKL09693	1451	1451/1451(100%) with no gaps	*B.yuanmingense* strain TTC4 or *B. liaoningense* strain LYG2

Table 4.5 indicated that the 5 NKL strains consisted of *B.yuanmingense* strain TTC4 or *B. liaoningense* strain LYG2 (NKL09216, NKL092231,NKL09666 and NKL09693) ; and one *B. elkanii* strain (NKL09273).

4.4.5.2 Identification of slow-growing soybean rhizobia using partial *dnaK* sequences

Figure 4.13 showed the isolated *dnaK* products of the five selected soybean rhizobial strains. The products were found to be the same size of approximatly 370 bp.

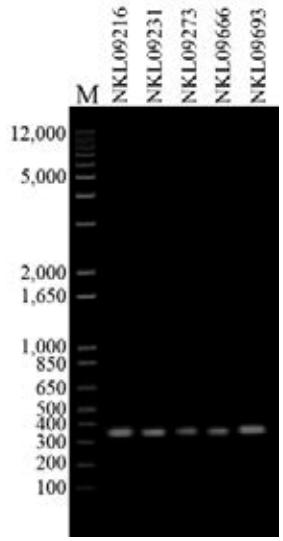


Figure 4.13 Isolated *dnaK* products of the five selected soybean rhizobial strains. All the isolated PCR products were found to be approximately 370 bp

Table 4.5 showed identification of the 5 slow-growing soybean rhizobia NKL strains by using the Blast program to compare the obtained partial *dnaK* sequences with those sequences in the GenBank database. The Blast program indicated the following results for PCR products of *dnaK* of the NKL strains:

NKL09216 (determined length 326 bp) could be related to *Bradyrhizobium yuanmingense* strain SR33 or *B.*yuanmingense strain SR88 with identities = 315/323(98%), gap = 3/323.

NKL09231 (determined length 348 bp) could be distantly related to either *Bradyrhizobium yuanmingense* strain SR33 or *B.*yuanmingense strain SR88 with identities = 292/322(91%), gap = 23/322. Since the percent homology was found to be less than 99%, NKL09231 could not be identified as *B.yuanmingense*.

NKL09273 (determined length 341 bp) could be distantly related to *Bradyrhizobium elkanii* strain USDA 46 with identities = 309/345(90%), gap = 22/345. Strain NKL09273 could not be identified as *B.elkanii* USDA46 because the percent homology was much less than 99%.

NKL09666 (determined length 327 bp) could be distantly related to *Bradyrhizobium yuanmingense* strain SR94 with identities = 313/323(97%), gap = 2/323.
NKL09693 (determined length 312 bp) could be distantly related to *Bradyrhizobium yuanmingense* strain SR33 or *B.yuanmingense* strain SR88 with identities = 287/295(97%), gap = 7/295.

Table 4.6 Summary of identification of the 5 slow-growing soybean rhizobia NKL strains based on partial *dnaK* sequences.

Strain	Size of *dnaK* product (bp)	Percent homology with sequences in GenBank	Identification
NKL09216	326	315/323(98%) with 3 gaps	Could not be identified
NKL09231	348	292/322(91%) with 23 gaps	Could not be identified
NKL09273	341	309/345(90%) with 22 gaps	Could not be identified
NKL09666	327	13/323(97%) with 2 gaps	Could not be identified
NKL09693	312	287/295(97%) with 7 gaps	Could not be identified

Table 4.6 indicated that no identification was obtained from partial sequnces of *dnaK* of the 5 NKL strains because the percent homologies were found to be less than 99%.

4.4.5.3 Identification of slow-growing soybean rhizobia using partial sequences of *glnII*

Figure 4.14 showed the isolated *glnII* products of the five selected soybean rhizobial strains. The products of strains NKL09216, NKL09231, NKL09666, and NKL09693 were found to be approximately 400 bp while that of strain NKL09273 was found to be approximately 500 bp. The reason was because a new set of forward and reverse primers had to be designed for use in the PCR amplification of *glnII* product when chromosomal DNA of strain NKL09273 was used as the target DNA. The previously-designed set of primers could anneal to the chromosomal DNA of the remaining four selected strains.

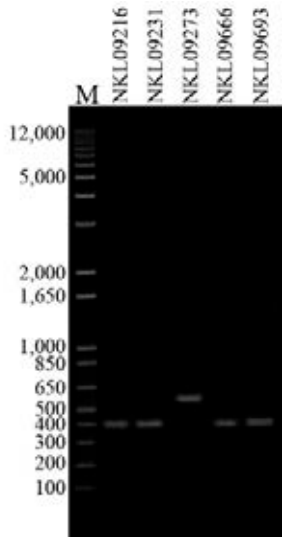


Figure 4.14 Isolated *glnII* products of the five selected soybean rhizobial strains. *glnII* product of strain NKL09273 was obtained with a different set of forward and reverse primer as explained in the text.

Table 4.7 showed identification of the 5 slow-growing soybean rhizobia NKL strains by using the Blast program to compare the obtained partial sequences of *glnII* with those sequences in the GenBank database. The Blast program indicated the following results from partial sequences of *glnII* of the five soybean rhizobial NKL strains:

NKL09216 (determined length 406 bp) could be related to *Bradyrhizobium yuanmingense* strain CCBAU 45370 with identities = 405/407(99%), gap = 1/407.
NKL09231 (determined length 408 bp) could be related to *Bradyrhizobium yuanmingense* strain CCBAU 45370 with identities = 404/408(99%), gap = 1/408.
NKL09273 (determined length 509 bp) could be related to *Bradyrhizobium elkanii* CCBAU 23090 or *B.elkanii* strain BuMiT9 or *B.elkanii* strain USDA46 with identities = 507/509(99%), gap = 0/509.
NKL09666 (determined length 407 bp) could be related to *Bradyrhizobium yuanmingense* strain CCBAU 45534 or *B.yuanmingense* strain CCBAU25575 or *B.yuanmingense* strain CCBAU 4551 or *B.yuanmingense* strain CCBAU051018 or *B.yuanmingense* strain CCBAU05623 or *B.yuanmingense* strain CCBAU10040 or *B.yuanmingense* CCBAU43003 or *B.yuanmingense* SR135 with identities = 406/407(99%), gap = 0/407.

NKL09693 (determined length 401 bp) could be distantly related to *Bradyrhizobium yuanmingense* strain SR42 or *B.yuanmingense* strain SR33 or *B.yuanmingense* strain SR88 with identities = 391/408(96%), gap = 8/408.

Table 4.7 Summary of identification of 5 slow-growing soybean rhizobium NKL strains based on partial sequences of *glnII.*

Strain	Size of *glnII* product (bp)	Percent homology with sequences in GenBank	Identification
NKL09216	406	405/407(99%) with 1 gap	*Bradyrhizobium yuanmingense* strain CCBAU 45370
NKL09231	408	404/408(99%) with 1 gap	*B.yuanmingense* strain CCBAU 45370
NKL09273	509	507/509(99%) with no gap	*B. elkanii* strains CCBAU or BuMiT9 or USDA46
NKL09666	407	406/407(99%) with no gap	*B.yuanmingense* strain CCBAU25575 or strain CCBAU4551 or strain CCBAU051018 or strain CCBAU05623 or strain CCBAU10040 or *strain* CCBAU43003 or *B.yuanmingense* SR135
NKL09693	401	391/408(96%) with 8 gaps	Could not be identified

Table 4.7 indicated that the 5 NKL strains consisted of *B.yuanmingense* strains (NKL09216, NKL092231,NKL09666 and NKL09693) and *B. elkanii* strain (NKL09273). In addition, there were partial sequences of *glnII* of about 8 *B.yuanmingense* strains in the GenBank database.

4.4.5.4 Identification of slow-growing soybean rhizobia by using partial *nifH* sequences.

Figure 4.15 showed the isolated PCR products of *nifH* of the five soybean rhizobial strains with the same size of approximately 360 bp.

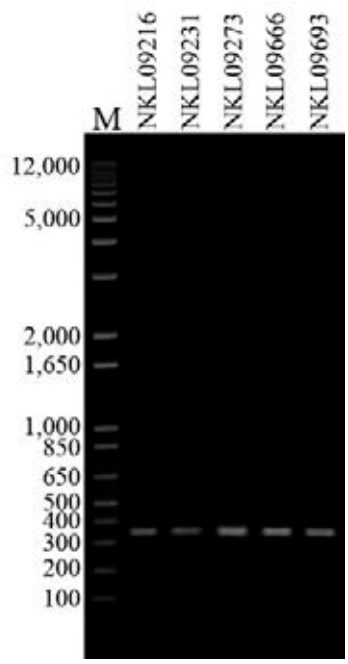


Figure 4.15 Isolated *nifH* products of the five selected soybean rhizobial strains.

Table 4.8 showed identification of the 5 slow-growing soybean rhizobia NKL strains by using the Blast program to compare the obtained partial *nifH* sequences with those sequences in the GenBank database. The Blast program indicated the following results for products of *nifH* of the five selected NKL strains:

NKL09216 (determined length 364 bp) could be related to *Bradyrhizobium yuanmingense* strain JNVU TF17 or *B.yuanmingense* strains SR42 or *B.yuanmingense* strain CCBAU65826 with identities = 362/364(99%), gap = 0/364.

NKL09231 (determined length 363 bp) could be related to *Bradyrhizobium yuanmingense* strain JNVU TF17 or *B.yuanmingense* strains SR42 or *B.yuanmingense* strain CCBAU65826 with identities = 361/364 (99%), gap = 1/364.

NKL09273 (determined length 364 bp) could be related to *Bradyrhizobium elkanii* strain S127 with identities = 359/364 (99%), gap = 0/364.

NKL09666 (determined length 366 bp) could be related to *Bradyrhizobium yuanmingense* strain JNVU TF17 or *B.yuanmingense* strains SR42 or *B.yuanmingense* strain CCBAU65826 with identities = 361/366 (99%), gap = 2/366.

NKL09693 (determined length 364 bp) could be related to *Bradyrhizobium yuanmingense* strain JNVU TF17 or *B.yuanmingense* strains SR42 or *B.yuanmingense* strain CCBAU65826 with identities = 361/364 (99%), gap = 0/364.

Table 4.8 Summary of identification of the 5 slow-growing soybean rhizobia NKL strains based on partial sequences of *nifH.*

Strain	Size of *nifH* product (bp)	Percent homology with sequences in GenBank	Identification
NKL09216	364	362/364(99%) with no gap	*Bradyrhizobium yuanmingense* strains JNVUTF17 or SR42 or CCBAU65826
NKL09231	363	361/364 (99%) with 1 gap	*Bradyrhizobium yuanmingense* strains JNVUTF17 or SR42 or CCBAU65826
NKL09273	364	359/364 (99%) with no gap	*B. elkanii* strain S127
NKL09666	366	361/366 (99%) with 2 gaps	*Bradyrhizobium yuanmingense* strains JNVUTF17 or SR42 or CCBAU65826
NKL09693	364	361/364 (99%) with no gap	*Bradyrhizobium yuanmingense* strains JNVUTF17 or SR42 or CCBAU65826

Table 4.8 indicated that the 5 NKL strains consisted of *B.yuanmingense* strains (NKL09216, NKL092231, NKL09666 and NKL09693) ; and one *B. elkanii* strain (NKL09273). Partial sequences of *nifH* of *B.yuanmingense* indicated that the four selected soybean rhizobia were the same strains JNVUTF17 or SR42 or CCBAU 65826.

4.4.5.5 Identification of slow-growing soybean rhizobia using *recA* sequences

Figure 4.16 showed the isolated *recA* products of the five selected soybean rhizobial strains. The products were approximately the same size of 260 bp.

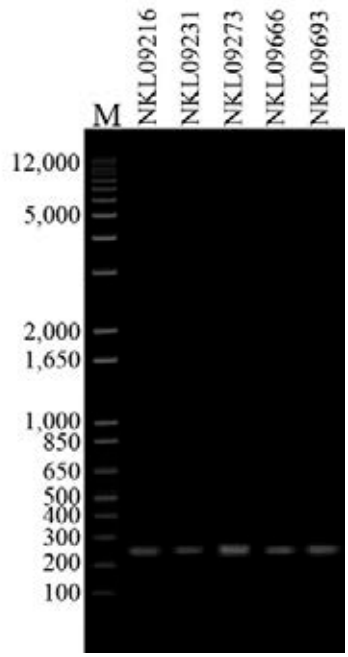


Figure 4.16 Isolated products of *recA* from the amplification of chromosomal DNA of the five selected soybean rhizobial strains.

Table 4.9 showed identification of the 5 slow-growing soybean rhizobia NKL strains by using the Blast program to compare the obtained partial *recA* sequences with those sequences in the GenBank database. The Blast program indicated the following results for products of *recA* of the five selected NKL strains:

NKL09216 (determined length 258 bp) could be related to *Bradyrhizobium yuanmingense* strain CCBAU 45370 or *B.yuanmingense* strain SR88 with identities = 257/259(99%), gap = 2/259.

NKL09231 (determined length 258 bp) could be distantly related to *Bradyrhizobium yuanmingense* strain CCBAU 45370 or *B.yuanmingense* strain SR88 with identities = 255/259(98%), gap = 2/259.

NKL09273 (determined length 262 bp) could be *Bradyrhizobium elkanii* strain Pop306 or *B. elkanii* strain SBR2B or *B. elkanii* strain SBR5A or *B. elkanii* strain SBR5B

or *B. elkanii* strain SBR7A or *B. elkanii* strain SBR8A or *B. elkanii* strain SBR8B or *B. elkanii* strain SBR8C with identities = 248/258(96%), Gaps = 2/258.

NKL09666 (determined length 258 bp) could be related to *Bradyrhizobium yuanmingense* strain CCBAU 65799 with identities = 210/212(99%), gap = 1/212.

NKL09693 (determined length 260 bp) could be related to *Bradyrhizobium yuanmingense* strain SR42 or *B.yuanmingense* strain SR33 or *B.elkanii* strain BuMiT9 with identities = 259/259(100%), gap = 0/259.

Table 4.9 Summary of identification of the 5 slow-growing soybean rhizobia NKL strains based on *recA* sequences.

Strain	Size of *recA* product (bp)	Percent homology with sequences in GenBank	Identification
NKL09216	258	257/259(99%) with 2 gaps	*Bradyrhizobium yuanmingense* strains CCBAU45370 or SR88
NKL09231	258	255/259(98%) with 2 gaps	Could not be identified
NKL09273	262	248/258(96%), with 2gaps	Could not be identified
NKL09666	258	210/212(99%) with 1 gap	*Bradyrhizobium yuanmingense* strains CCBAU45370 or SR88
NKL09693	260	259/259(100%) with no gap	*B.yuanmingense* strains SR42 or SR33 or *B.elkanii* strain BuMiT9

Table 4.9 indicated that the 5 NKL strains consisted of some *B.yuanmingense* strains (NKL09216, NKL09666 and NKL09693) while strains NKL09231 and NKL09273 could not be identified due to relatively low percent homology with sequnces in the GenBank database. It is noted that only the partial sequence of *recA* of strain NKL09693 suggested this strain could be *B.elkanii* strian BuMiT9 while partial sequence of other gene such as *nifH* revealed the strain was closely related to *B.yuanmingense*.

4.4.5.6 Dendrograms for the identification of the five selected slow-growing soybean rhizobia by using partial sequences of 16S rDNA, *dnak, glnII, nifH* and *recA*

Figures 4.17-4.22 showed phylogenetic trees or dendrograms obtained from partial nucleotide sequences of 16S rDNA, *dnaK, nifH, glnII, recA* and concatenated partial sequences of the 5 genes of the five selected soybean rhizobial strains and some reference strains. The boot strap numbers were found to be satisfactory in some nodes and unsatisfactory in other nodes of the trees or dendrograms. Other methods for constructing the dendrograms were used such as the Maximum likelihood method, the Neighbor-joining method with less satisfactory results as showen in Appendix J. However, when UPGMA method was used to construct all the dendrograms, the results were relatively satisfactory because *Caulobacter crescentus* CB15 which was used as the outgroup was found to be distantly related to all the soybean rhizobial strains used in the construction of the dendrograms. In addition, the bootstrap numbers were mostly in the acceptable range which was close to 100 for most of the dendrograms' nodes.

Figure 4.17 showed the dendrogram obtained by using partial sequences of 16S rDNA could not delineate the five selected soybean rhizobial strains into different species of *Bradyrhizobium* spp. Instead, the five selected strains were found to be in the same cluster which was related to *B.elakanii, B.japonicum* and relatively distantly related to *B.yuanmingense* and *B.liaoningense*. Previously Blast results of partial sequences of 16S rDNA, *dnaK, glnII, nifH,* and *recA* did not reveal any of the five selected strains as *B.japonicum*.

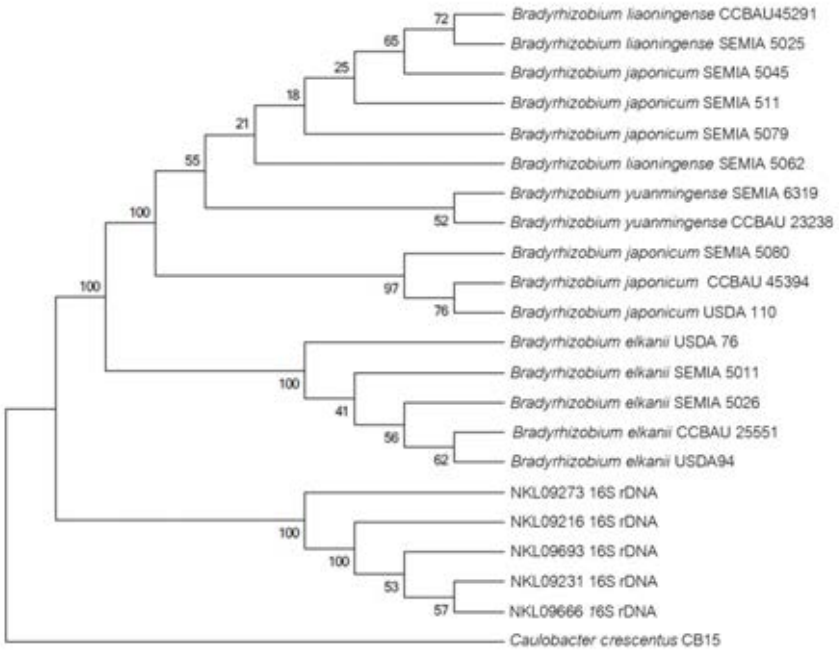


Figure 4.17 UPGMA dendrogram constructed from partial nucleotide sequences of 16S rDNA of soybean rhizobial strains. *Caulobacter crescentus* CB15 was used as the outgroup.

Figure 4.18 showed UPGMA dendrogram constructed with partial sequences of *dnaK* of soybean rhizobial strains with *Caulobacter crescentus* CB15 as the outgroup. The results showed relatively satisfactory bootstrap numbers with distantly-related outgroup. Strain NKL09273 was found to be in the same cluster as *B.elkanii* . Strains NKL09216, NKL09231, and NKL09693 were found to be in the same cluster and were found to be closely related to *B.liaoningense* and *B.japonicum* USDA110. Strain NKL09666 was found to be related to *B.yuanmingense* strain CCBAU23238 and SR69

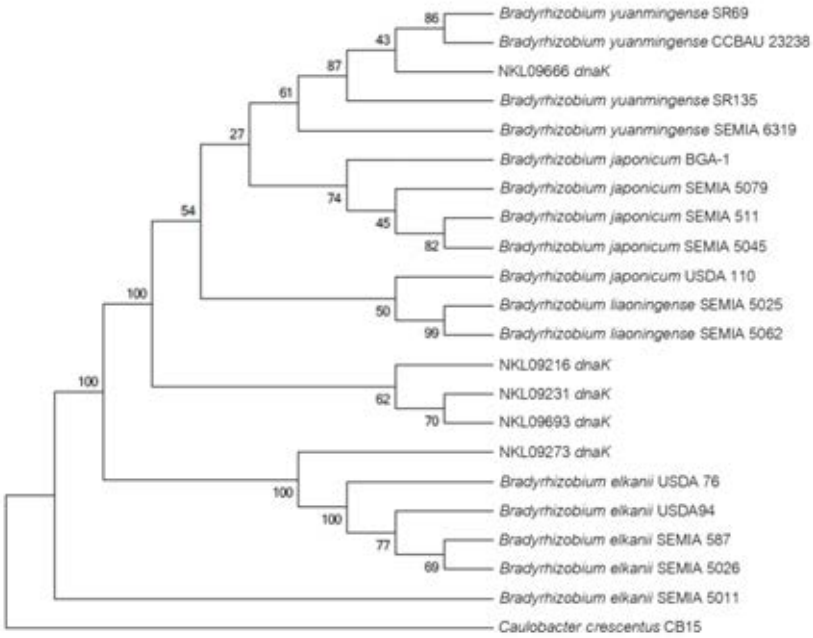


Figure 4.18 UPGMA dendrogram constructed from partial nucleotide sequences of *dnaK* of soybean rhizobial strains. *Caulobacter crescentus* CB15 was used as the outgroup.

Figure 4.19 showed UPGMA dendrogram constructed from partial nucleotide sequences of *nifH* of soybean rhizobial strains with the free-living N_2-fixaing *Azospirillum picis* DSM19922 as the outgroup because *Caulobacter crescentus* CB15 had no *nifH*. The dendrogram showed NKL09231 was related to *B. elkanii* strain USDA94 and *B. yuanmingense* CCBAU10071 with a relatively low bootstrap value of 44. In addition, strains NKL09666, NKL09216, NKL09693 and NKL09273 were closely related to *B. yuanmingense* SR135.

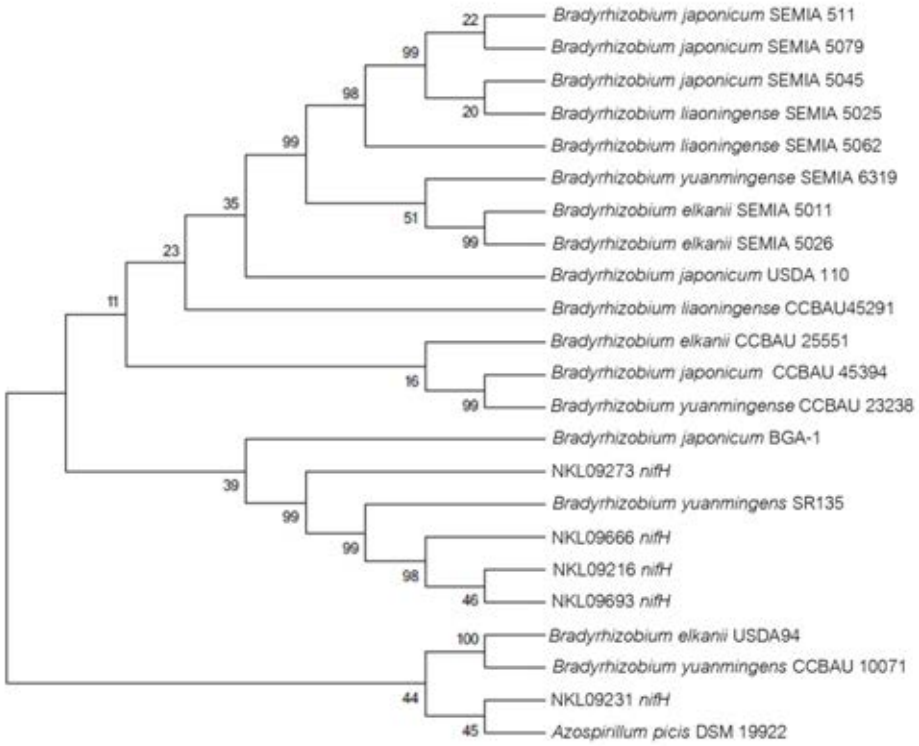


Figure 4.19 UPGMA dendrogram constructed from partial nucleotide sequences of *nifH* of soybean rhizobial strains. *Azospirillum picis* DAM 19922 was used as the outgroup.

The dendrogram constructed from partial sequences of *glnII* as shown in Figure 4.20 showed strain NKL09693 was related to *B.yuanmingense* SEMIA6319, while the three strains NKL09216, NKL09231, and NKL09666 were related to *B.yuanmingense* SR135, strain NKL09273 was found to be related to four strains of *B.elkanii*. The outgroup *Caulobacter crescentus* CB15 was found to be distantly related to the soybean rhizobial strains.

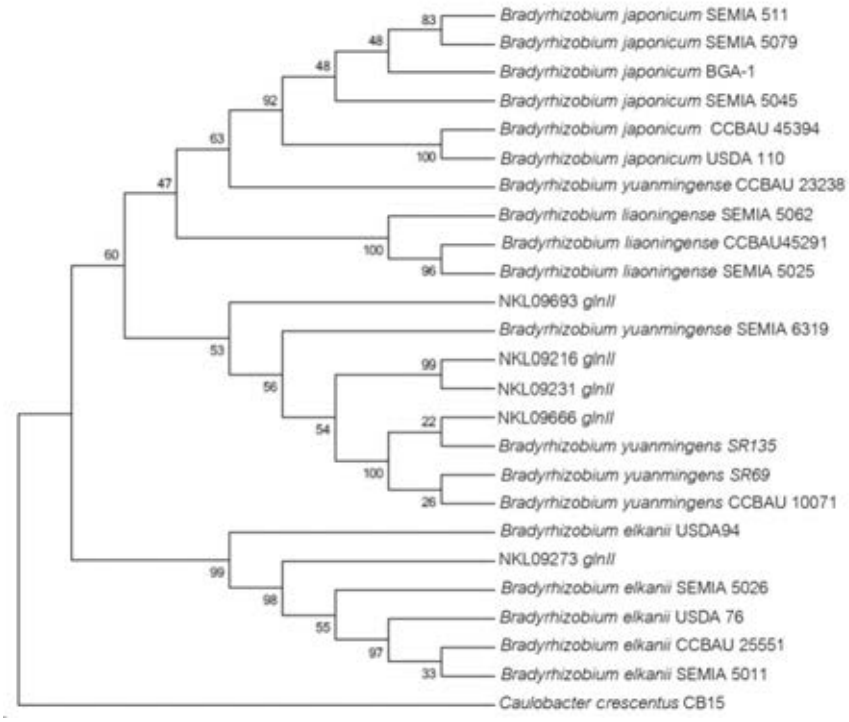


Figure 4.20 UPGMA dendrogram constructed from partial nucleotide sequences of *glnII* of soybean rhizobial strains. *Caulobacter crescentus* CB15 was used as the outgroup.

Figure 4.21 showed the UPGMA dendrogram obtained by using partial sequences of *recA.* In this dendrogram, the outgroup *Caulobacter crescentus* CB15 *recA* partial sequence was found to be relatively closely related to those of rhizobial strains. The reason is because *recA* which encodes an enzyme involved in homologous recombination, as briefly explained in the Literature Survey section of the thesis, is an important enzyme in micro-organisms and hence the sequences would be well-preserved. The results as shown in Table 4.10 showed all the 5 selected rhizobial strains were related to *B.japonicum* strain CCBAU 45394 and 45291.

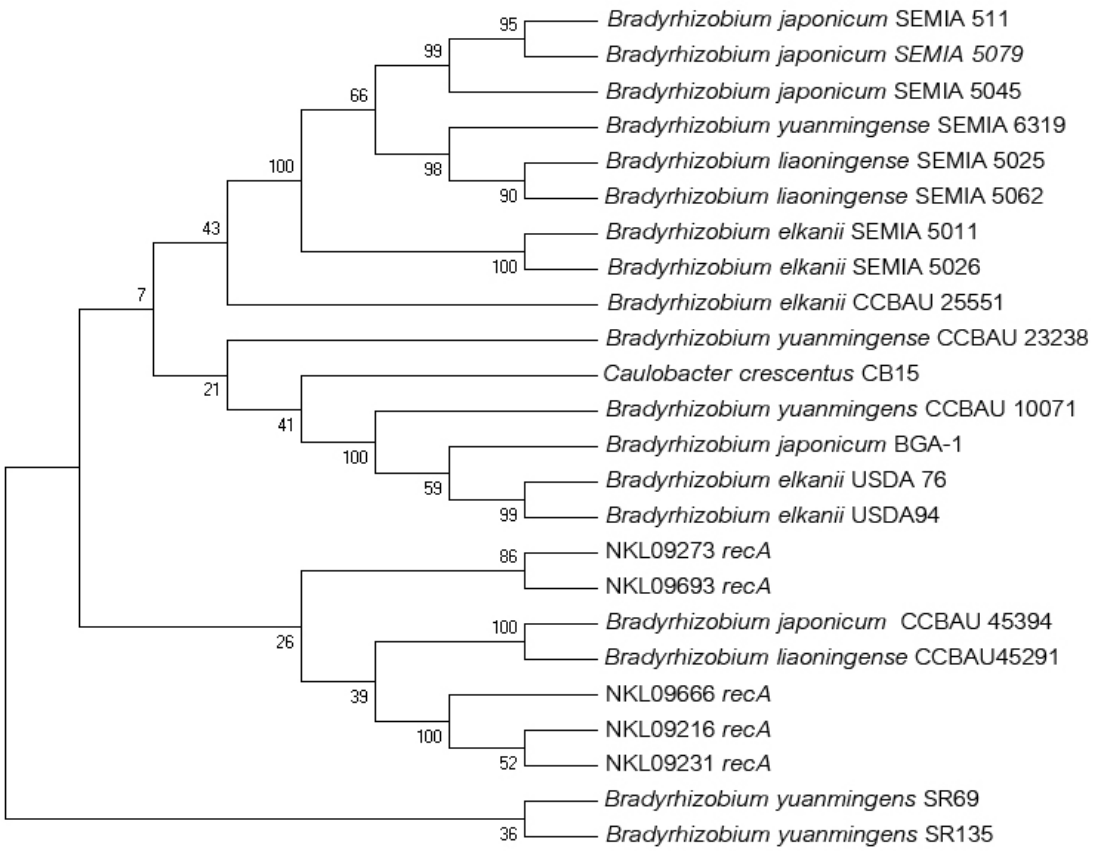


Figure 4.21 UPGMA dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of *recA.*

Figure 4.22 showed a dendrogram constructed with concatenated partial sequnces of the five genes (16S rDNA, *dnaK, glnII, recA,* and *nifH*). There are no outgroup since *Caulobacter crescentus* CB15 did not contain *nifH*. The results as shown in Table 4.10 indicated that NKL09216, NKL09231, NKL09666, and NKL09693 were closely related with the bootstrap numbers of 100 and these four strains were found to be related to *B.elkanii* strain USDA94, *B.yuanmingense* strains CCBAU10071 and SR135, *B.japonincum* strains USDA110, although the bootstrap numbers were low. Moreover, the results showed strain NKL09273 was related to *B.elkanii* strain CCBAU25551 and *B.yuanmingense* strain CCBAU23283.

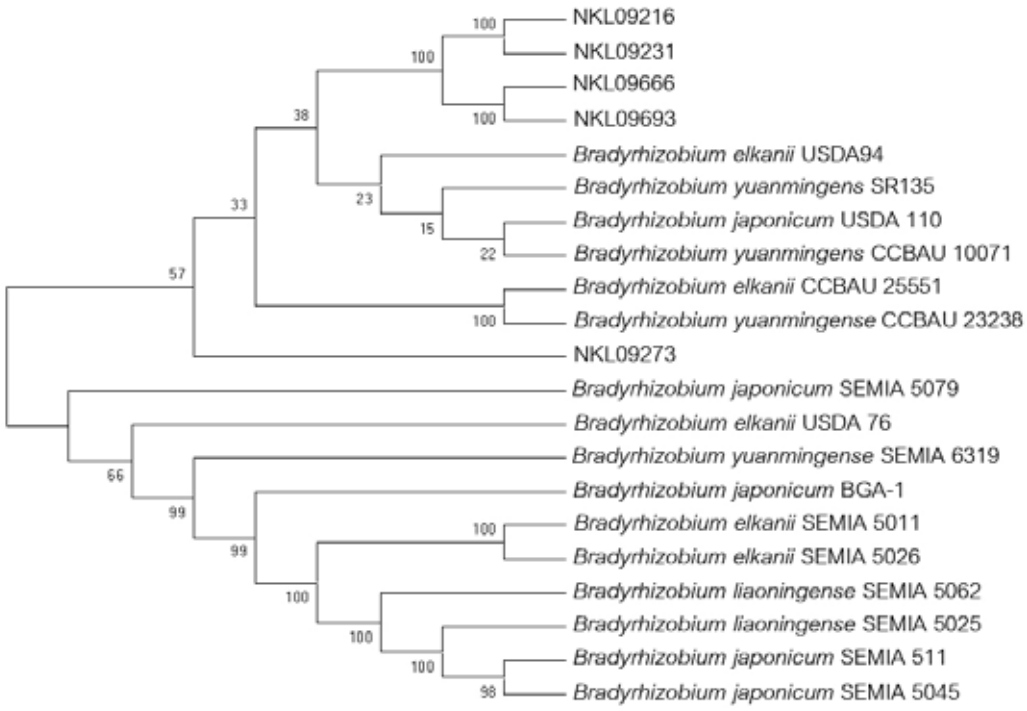


Figure 4.22 UPGMA dendrogram constructed from concatenated partial sequences of 16S rDNA-*dnaK- nifH- glnII- recA* of rhizobial strains

The UPGMA dendrogram constructed from concatenated partial sequences of 16S rDNA-*dnaK- nifH- glnII- recA* of rhizobial strains had no outgroup because *Caulobacter crescentus* CB15 does not contain *nifH.* The results showed bradyrhizobial strains were divided into two distinct clusters, the upper and the lower clusters. The lower cluster showed that the reference strains *B. japonicum, B. liaoningense,* and *B. elkanii* , were closely related with the bootstrap numbers of 100. On the contrary, the upper cluster showed less reliable results with the bootstrap numbers of less than 50 for the reference strains. However, the results showed that the four selected strains NKL09216, NKL09231, NKL09666, and NKL09693 formed a subcluster with very closely related phylogenetic relationships with the bootstrap numbers of 100. Strain NKL09273, though belonged to the upper cluster, was found to be in a separate subcluster. Although the bootstrap number of 99 was obtained for the reference strains *B.elkanii* strain CCBAU 25551 and *B. yuanmingense* strain CCBAU 23283 in the upper cluster, all the other nodes in the upper cluster were found with bootstrap numbers of less than 50. Hence, it was identified with less confidence that the four soybean rhizobial strains in the first subcluster were related to *B.elkanii* USDA94 and *B.yuanmingense* SR135 and *B.japonicum* USDA110 and *B.*

yuanmingense CCBAU10071 . Strain NKL09273 which was found in a separate subcluser in the upper cluster was found to be related to *B. elkanii* strain CCBAU25551 and *B. yuanmingense* strain CCBAU23283. Table 4.10 summarized the findings obtained from the dendrogram constructed with concatenated partial sequences of the five genes : 16S rDNA-*dnaK- nifH- glnII- recA* of rhizobial strains.

Table 4.10 Identification of five soybean rhizobial strains by using dendrograms constructed from CRL-7 RAPD-PCR fingerprints and Multilocus Sequence Analysis.

Strains	CRL-7 RAPD-PCR dendrogram	16S rDNA	*dnaK*	*glnII*	*nifH*	*recA*	Concatenated sequences
NKL09216	*B.elkanii*	*B.elkanii* or *B.japonicum* or *B. yuanmingense* or *B. liaoningense*	*B.japonicum* or *B. liaoningense*	*B.yuanmingense* SR135	*B.yuanmingense* SR135	*B.japonicum* CCBAU 45394 or *B. liaoningense* CCBAU 45291	*B.japonicum* USDA110 or *B.yuanmingense* SR135 and CCBAU10071 or *B.elkanii* USDA94
NKL09231	*B.liaoningense* or *B.elkanii*	*B.elkanii* or *B.japonicum* or *B. yuanmingense* or *B. liaoningense*	*B.japonicum* or *B. liaoningense*	*B.yuanmingense* SR135	*B.elkanii* USDA94 or *B. yuanmingense* CCBAU1071	*B.japonicum* CCBAU 45394 or *B. liaoningense* CCBAU 45291	*B.japonicum* USDA110 or *B.yuanmingense* SR135 and CCBAU10071 or *B.elkanii* USDA94
NKL09273	*B.elkanii*	*B.elkanii* or *B.japonicum* or *B. yuanmingense* or *B. liaoningense*	*B. elkanii*	*B. elkanii* (four strains)	*B.yuanmingense* SR135	*B.japonicum* CCBAU 45394 or *B. liaoningense* CCBAU 45291	*B.yuanmingense* CCBAU 23238 or *B.elkanii* CCBAU 25551
NKL09666	*B.elkanii* or *B.liaoningense*	*B.elkanii* or *B.japonicum* or *B. yuanmingense* or *B. liaoningense*	*B.yuanmingense*	*B.yuanmingense* SR135	*B.yuanmingense* SR135	*B.japonicum* CCBAU 45394 or *B. liaoningense* CCBAU 45291	*B.japonicum* USDA110 or *B.yuanmingense* SR135 and CCBAU10071 or *B.elkanii* USDA94
NKL092693	*B.yuanmingenese*	*B.elkanii* or *B.japonicum* or *B. yuanmingense* or *B. liaoningense*	*B.japonicum* or *B. liaoningense*	*B.yuanmingense* SEMIA6319	*B.yuanmingense* SR135	*B.japonicum* CCBAU 45394 or *B. liaoningense* CCBAU 45291	*B.japonicum* USDA110 or *B.yuanmingense* SR135 and CCBAU10071 or *B.elkanii* USDA94

CHAPTER V
DISSCUSSION

RAPD-PCR fingerprinting

In this research , identical RAPD-PCR fingerprints were used to initially group bacteria isolated from root nodules into the same strains. Primer RPO1 was chosen for use in the DNA fingerprinting because it annealled to the 20 conserved nucleotide sequence in the promoter of *nifH* of the fast-growing *Rhizobium trifolii* strains Rt 329, Rt RS1 and *R. meliloti* RmP1 (Schofield and Watson,1985). In addition, Richardson et al. (1995) reported that the primer RPO1 could be used in PCR-fingerprinting to differentiate among different strains of fast-growing *Rhizobium* spp. Primer RPO1 was chosen for use in this research because it was expected that the presence of a PCR product due to the extension of a DNA fragment after the annealling of the primer would be a confirmation for the presence of *nifH* which encodes the Fe protein subunit of the enzyme nitrogenase. In this research, RPO1-PCR fingerprinting was obtained at least twice. The PCR fingerprints were not always reproducible. Sometimes one PCR product band was obtained, other times more than one PCR product band were obtained for the same strains (results not shown).

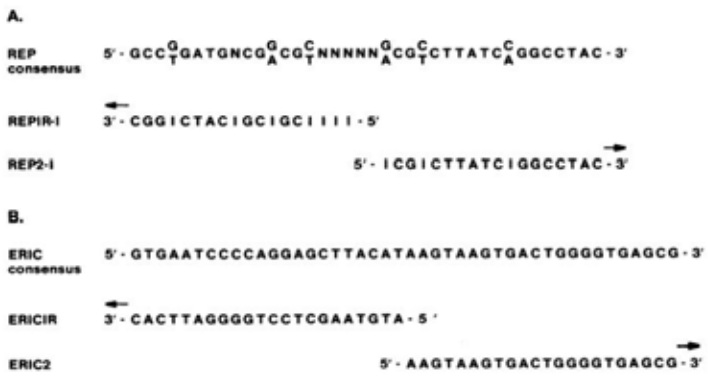


Figure 5.1 Nucleotide sequence of the REP and ERIC primers**.** (A) REP consensus sequence and nucleotide sequence of the two REP primers (REP1R-I and REP2-I), positioned relative to the REP consensus sequence. The I's denote inosines. (B) ERIC consensus sequence and nucleotide sequence of the two ERIC primers (ERIClR and ERIC2), positioned relative to the ERIC consensus sequence. The arrows denote the direction of *Taq* polymerase extension (de Bruijn, 1992).

De Bruijn (1992) reported that enteric bacteria such as *E. coli* and *Salmonella typhimurium* and several other Gram negative bacteria including *Bradyrhizobium* spp. contained several short intergenic repeated sequences with highly conserved central inverted repeats known as the repetitive extragenic palindromic elements (REPs) and the enterobacterial repetitive intergenic consensus (ERIC) sequences. When all the available REP and ERIC sequences were aligned, the REP and ERIC consensus sequences as shown in Figure 19 were obtained. These sequences have been used in PCR-DNA fingerprinting of several soybean rhizobia including the rhizobium strains used in the commercial production of inoculants in Spain (Binde et al. 2009). But for the purpose of grouping soybean rhizobia with identical fingerprints into the same strains which was part of the topic for this thesis, the use of RPO1 or CRL-7 as the primer in the PCR-DNA fingerprinting was satisfactory.

Bromothymol blue reactions in slow-growing soybean rhizobia

According to Somasegaran and Hoben (1994), the indicator dye bromothymol blue was green on an agar plate with YM medium (YMA) at pH 6.8. Slow-growing soybean rhizobia turn the color of bromothymol blue to blue due to the secretion of alkali product(s). Other researchers reported that fast-growing soybean rhizobia showed an acid bromothymol blue reaction while slow-growing soybean rhizobia showed an alkali bromothymol blue reaction (Alberton et al., 2006). In this research , it was not found out that two types of bromothymol blue reactions were found in slow-growing soybean rhizobia as reported by Maruekarajtinpleng (2010). The experimental results showed that during growth on YMA with bromothymol blue at the initial pH of 6.8, no soybean rhizobial strains secreted alkali product(s) which turned the medium blue throughout the 10-day incubation time. All the 43 isolated soybean rhizobial strains were found to secrete acidic product(s) during the first 5-day incubation and secrete acidic product(s) during the 10-day incubation.

Average total nitrogen (g/100g plant)

The average total nitrogen content of soybean would have been much more accurately expressed as the average of total nitrogen of soybean plant (g/100g plant) multiplied by the average dry weight of soybean in each Leonard jar (Wipa Homhual, personal communication).

Multilocus Sequence Analysis in the identification of five selected slow-growing soybean rhizobia

The average length of the isolated 16S rDNAs of the five selected soybean rhizobial strains was 1450 bp which was in the same range as those reported by Binde et al. (2009) and Menna et al. (2006). However, the length of the PCR products of the other four genes were about three times less than those reported in GenBank (results not shown). In retrospect, it was thought that use of the relatively short concatenated partial sequences of the genes might be one reason the dendrogram obtained from the concatenated sequences did not have bootstrap numbers higher than 50 as shown in the upper cluster in Figure 4.22. In addition, the shorter concatenated sequences used in the dendrogram construction might explain the high bootstrap number of 100 which showed a very close relationship amongst the four selected soybean rhizobial strains (NKL09216, NKL09231, NKL09666, and NKL09673) and a relatively distant relationship of the remaining strain NKL09273 which was found to belong to a separate subcluster in the upper cluster as shown in Figure 4.22. Taking into consideration the overall results obtained from the dendrograms of partial sequences of the genes including the use of the concatenated partial sequences as shown in Tables 4.9 and 4.10, the four strains in the first subcluster seemed to belong to either *B . japonicum* or *B. yuanmingense* ; and the strain NKL09273 seemed to be *B. elkanii.* It is very interesting to note that *B.japonicum* USDA110 could be detected in some of the selected strains since *B.japonicum* USDA110 was used in the rhizobium biofertilizer which was developed and distributed to farmers for use in 1960s (Wipa Homhaul, personal communication).

The results as shown in Tables 4.9 and 4.10 showed MLSA using either partial sequences of one or concatenated partial sequences of 16S rDNA-*dnaK-nifH-glnII-recA* genes could not differentiate amongst the 4 selected strains (NKL09216, NKL09231, NKL09666, and NKL09693). However, MLSA results could be used to state that strain NKL09273 was likely *B.elkanii* or *B.yuanmingense* . It is interesting to note that dendrograms constructed with either 16S rDNA or *dnaK* partial sequences revealed the four strains NKL09216, NKL09231, NKL09666, and NKL09293 could also be *B.liaoningense*. It can be noted from the results that the design of primers for the amplification of genes for use in MLSA is very important. If primers yield short products of genes which are relatively well-conserved, such as 16S rDNA. *glnII, recA, nifH* for use in MLSA, the ability to resolve various species into distinct species may not be possible.

In 2008, Vinuesa et al used partial sequences of *atp D, recA, glnII* and *rhoB* to resolve 76 Bradyrhizobial strains isolated from the nodules of *Glycine max* trap plants inoculated with soil samples from Myanmar, India, Nepal, and Vietnam. In this case MLSA could resolve all the 76 strains which were used with 33 reference strains into the four-slow growing soybean rhizobia. Hence, It is recommended from the results of this thesis that new primers for the amplification of longer PCR products and more isolated strains as well as reference strains be used in future research on the use of MLSA in soybean rhizobial taxonomy.

CHAPTER VI
CONCLUSION

In this research 150 bacterial isolates obtained from root nodules of soybean cv. Chiangmai 2 grown in a 15 x 24 m^2 experimental plot in Nongkula subdistrict, Phitsanulok province, Thailand, were categorized as fast- or slow-growing isolates. A total of 116 slow-growing bacterial isolates were obtained for RAPD-PCR fingerprinting using either RPO1 or CRL-7 as the primer. 43 different strains were obtained by grouping bacterial isolates into the same strains. Authentication tests showed the 43 slow-growing bacterial strains were soybean rhizobia with total nitrogen of the whole plant as determined by the Keldjahl method ranging from 0.1 to 1.0 g/100 g plant. Two dendrograms were constructed with either RPO1-RAPD-PCR fingerprints or CRL-7-RAPD-PCR fingerprints. Five soybean rhizobial strains were selected for use in polyphasic taxonomy and Multilocus Sequence Analysis using partial sequences and concatenated partial sequences of the following five genes: 16S rDNA, *dnak, nifH, glnII*, and *recA.* The five selected soybean rhizobia were found to have very small colonies of less than 0.1 mm when grown on plates containing YM medium with congo red. All the five strains were found to secrete acidic products when grown on YM containing bromothymol blue agar plates. Each of the selected soybean rhizobial strains was found to have one subpolar flagellum as revealed by negative staining and observing under the Transmission Electron Microscope. Using the BLAST program to compare homology between the obtained partial sequences of each gene with those deposited in GenBank as well as using dendrograms constructed from partial sequences of each of the following five genes and the partial concatenated sequences to construct dendrograms revealed that NKL09216, NKL09231, NKL09666, and NKL09693 were closely related to each other with bootstrap numbers of 100 and they could be either *B. japonicum,* or *B. yuanmingense* while the other selected soybean rhizobial strain was found to belong to a separate subset and could be *B. elkanii.* It is suggested from the results of this research that primers should be designed to yield large PCR products for longer nucleotide sequences for use in MLSA . Use of a large number of isolated strains and

type and references strains should also be used to construct dendrograms that could resolve soybean rhizobia into species or strain levels.

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APPENDICES

APPENDIX A

BACTERIAL GROWTH MEDIA AND PLANT NUTRIENT SOLUTIONS

Preparation of all bacterial growth media and plant nutrient solutions are as described by Somasegaran and Hoben (1994) unless otherwise stated.

Yeast Extract Mannitol Broth (YMB)

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
Deionized water	1.0 liter

pH of medium was adjusted to 6.8 with 0.1 N NaOH The medium was autoclaved at 121°C for 15 min.

Yeast Extract Mannitol Agar (YMA)

YMB	1 liter
Agar	15 g

Agar was added to 1 liter of YMB. The solution was shaken to suspend the agar then autoclaved at 121°C for 15 min. After autoclaving, the medium was shaken to ensure even mixing of melted agar with medium before pouring onto petri dishes and left to solidify.

YMA with Congo Red

Congo Red stock solution: 250 mg of Congo Red dissolved in 100 ml of deionized water. 10 ml of Congo Red stock solution were added to 1 liter of YMA. The final Congo Red concentration was 25 $\mu g.ml^{-1}$. The medium was autoclaved at 121°C for 15 min.

YMA with Bromthymol Blue (BTB YMA)

Bromthymol Blue stock solution: 0.5 g of Bromthymol Blue were dissolved in 100 ml of ethanol. 5 ml of Bromthymol Blue stock solution were added to 1 liter of YMA. The final Bromthymol Blue concentration was 25 $\mu g.ml^{-1}$. The medium was autoclaved at 121°C for 15 min.

N-free Nutrient Solutions

Stock Solutions	Chemicals	g/liter
1	$CaCl_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$FeC_6H_5O_7.3H_20$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2\ MoO_2.7\ H_2O$	0.048

Warm water was used to prepare stock solutions to get the ferric-citrate into solution. Ten liters of full-strength plant culture solution were prepared as follows:

- To 5 liters of water, add 5 ml of each stock solution and mix,
- Adjust pH to 6.8 with 1 N HCl
- Dilute to 10 liters by adding water
- For nutrient solution, 0.05% KNO_3 was added to give final N concentration of 70 ppm.

APPENDIX B

CHEMICALS AND SOLUTIONS

1. Solutions for DNA extraction

Saline-EDTA solution

15 mM NaCl, 10 mM EDTA, pH 8.0

0.09 g NaCl, 0.29 g EDTA

were added to distilled water. The final volume was made to 100 ml. 0.1 N NaOH was used to adjust pH to 8.0 before autoclaving at 121°C for 15 min.

DNAzol

DNAzol solution (Molecular Research Lab, MRL) was used according to the manufacturer's instruction.

2. Electrophoresis Buffer

50X Tris Acetate Buffer (TAE buffer)

Tris base	242	g.
glacial acetic acid	57.1	ml
0.5 M EDTA pH 8.0	100	ml

were added to double distilled water. 6 N HCl was used to adjust pH to 8.0. The final volume was added to 1000 ml.

APPENDIX C

GROUPING OF BACTERIAL ISOLATES WITH IDENTICAL RAPD-PCR FINGERPRINTS INTO THE SAME STRAINS

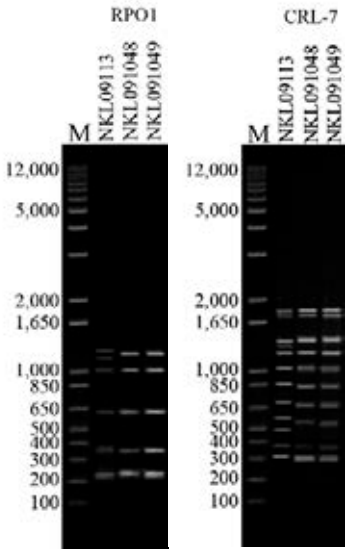


Figure C.1 The same strain:
NKL09113=NKL091048=NKL091049.

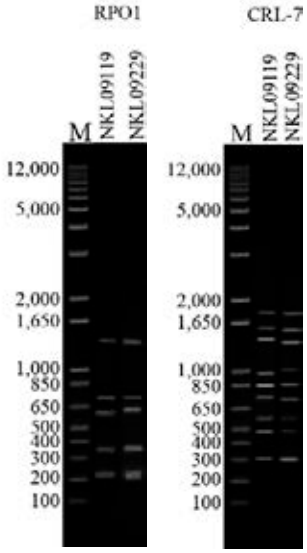


Figure C.2 The same strain:
NKL09119=NKL09229.

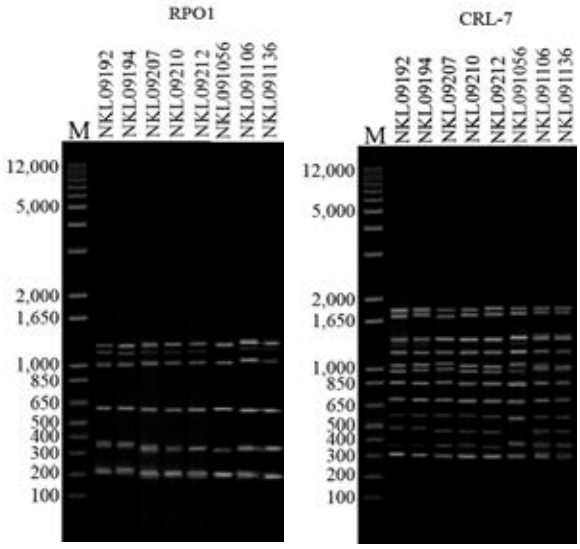


Figure C.3 The same strain:
NKL09192=NKL09194=NKL09207=NKL09210=
NKL09212=NKL091056=NKL091106=NKL091136.

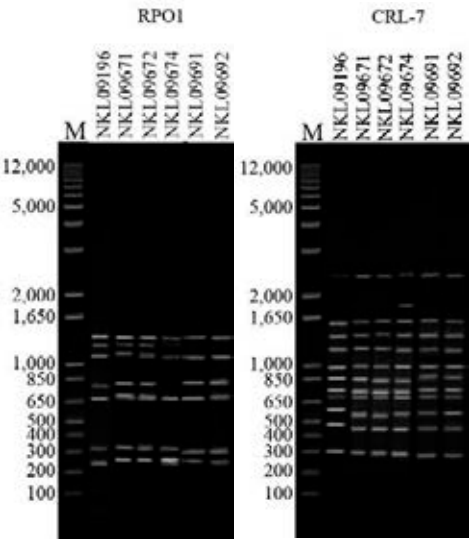


Figure C.4 The same strain:
NKL09196=NKL09671=NKL09672=
NKL09674=NKL09691=NKL09692.

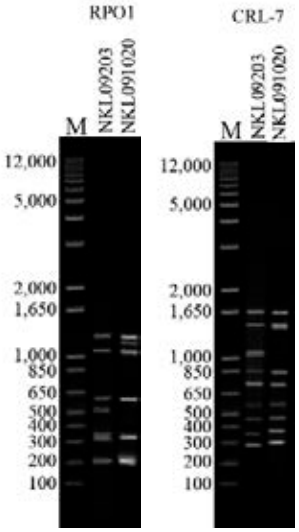


Figure C.5 The same strain: NKL09203=NKL091020.

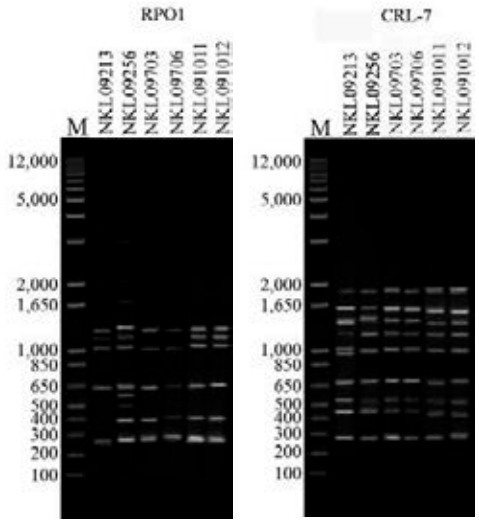


Figure C.6 The same strain: NKL09213=NKL09256=NKL09703= NKL09706=NKL091011=NKL091012.

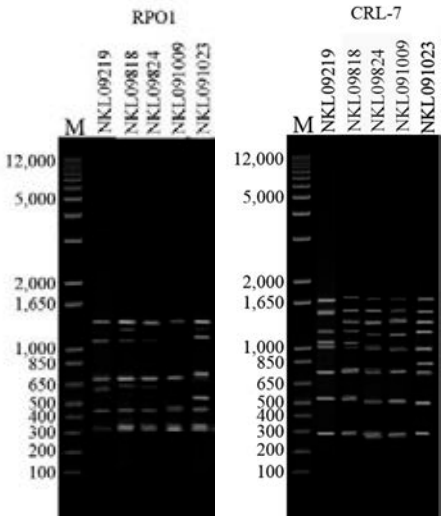


Figure C.7 The same strain: NKL09219=NKL09818=NKL09824= NKL091009=NKL091023.

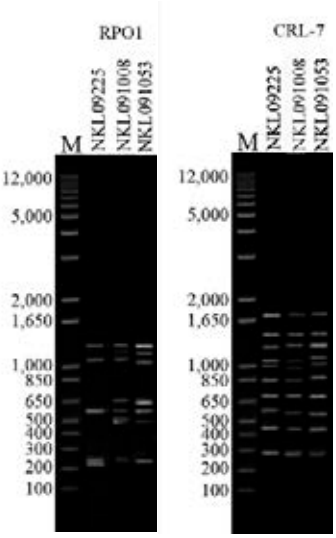


Figure C.8 The same strain: NKL09225=NKL091008=NKL091053.

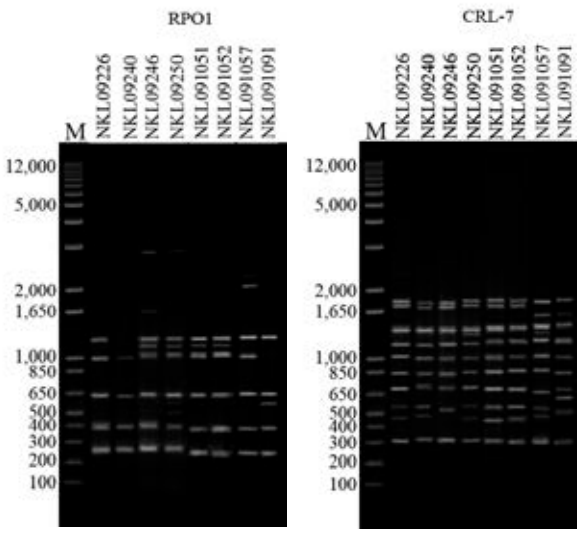


Figure C.9 The same strain:
NKL09226=NKL09240=NKL09246=NKL09250=
NKL091051=NKL091052=NKL091057=NKL091091.

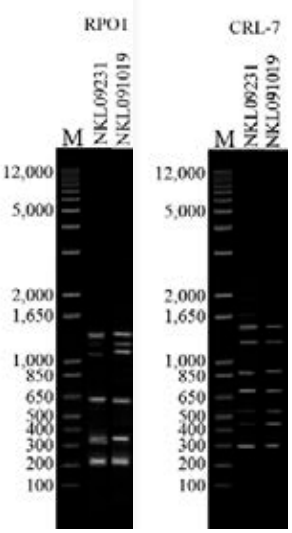


Figure C.10 The same strain:
NKL09231=NKL091019.

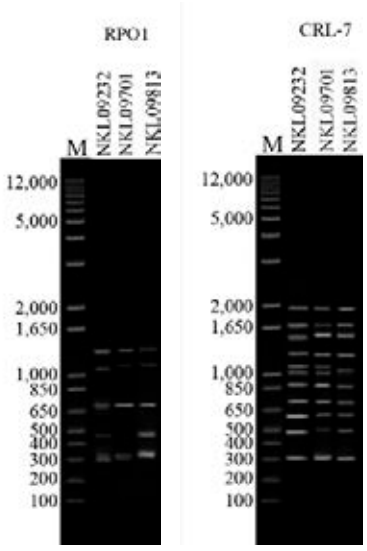


Figure C.11 The same strain:
NKL09232=NKL09701=NKL09813.

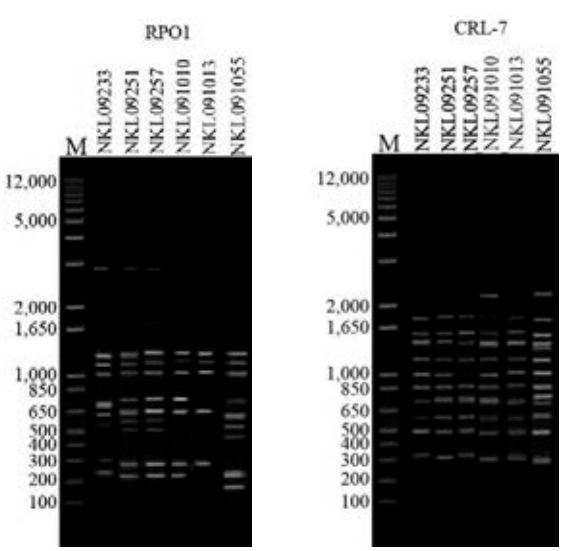


Figure C.12 The same strain:
NKL09233=NKL09251=NKL09257=
NKL091010=NKL091013=NKL091055.

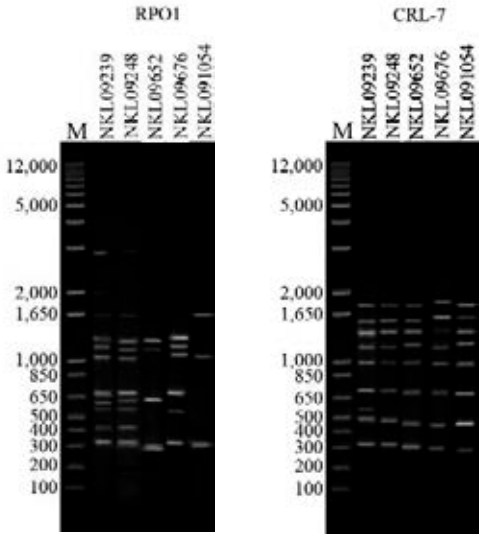


Figure C.13 The same strain: NKL09239=NKL09248=NKL09652 =NKL09676=NKL091054.

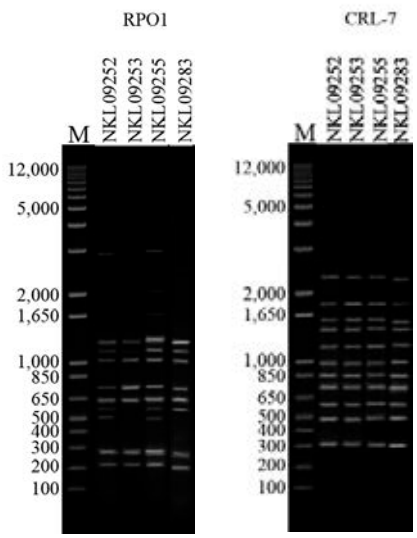


Figure C.14 The same strain: NKL09252=NKL09253=NKL09255 =NKL09283.

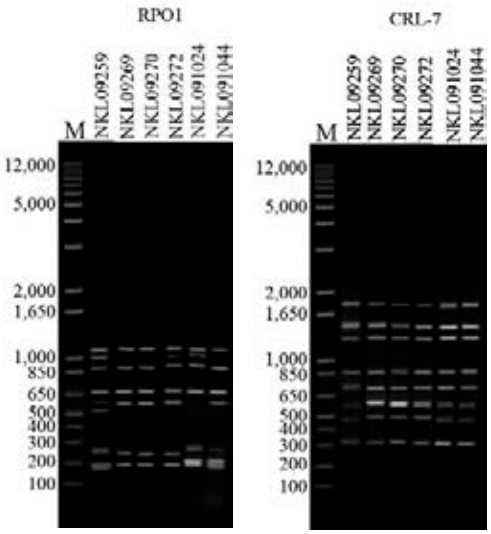


Figure C.15 The same strain: NKL09259=NKL09269=NKL09270= NKL09272=NKL091024=NKL091044.

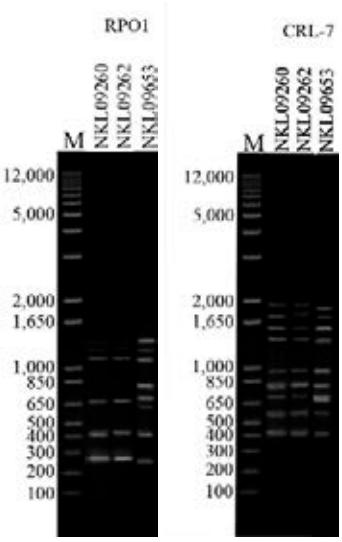


Figure C.16 The same strain: NKL09260=NKL09262=NKL09653.

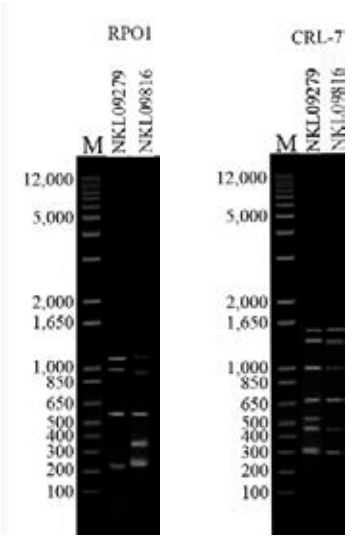


Figure C.17 The same strain: NKL09279=NKL09816.

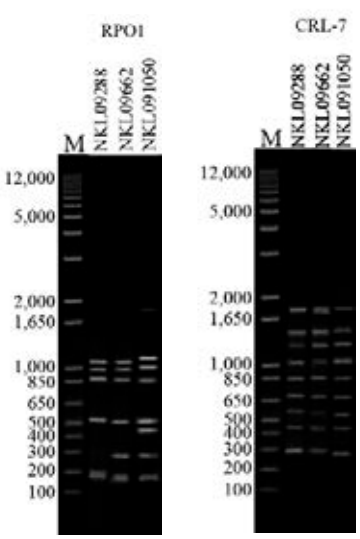


Figure C.18 The same strain: NKL09288=NKL09662=NKL091050.

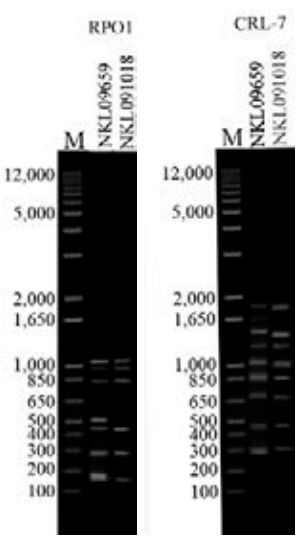


Figure C.19 The same strain: NKL09659=NKL091018.

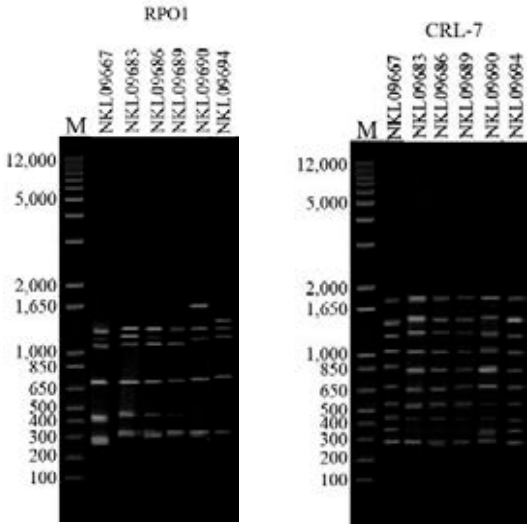


Figure C.20 The same strain: NKL09667=NKL09683=NKL09686= NKL09689=NKL09690=NKL09694.

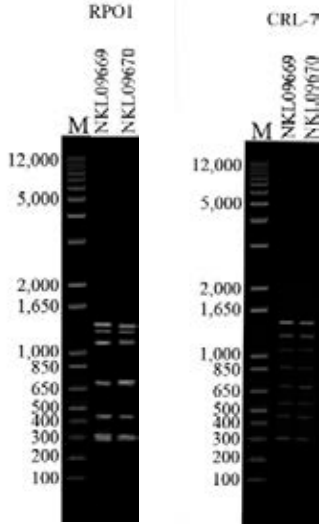


Figure C.21 The same strain: NKL09669=NKL09670.

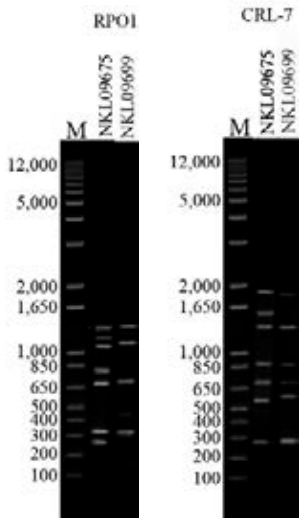


Figure C.22 The same strain: NKL09675=NKL09699.

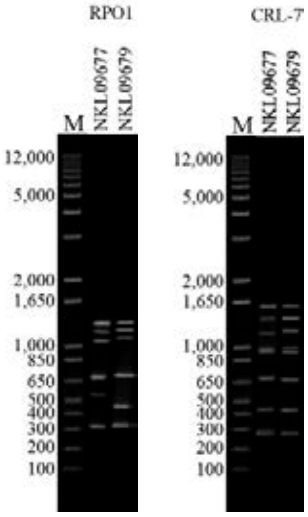


Figure C.23 The same strain: NKL09677=NKL09679.

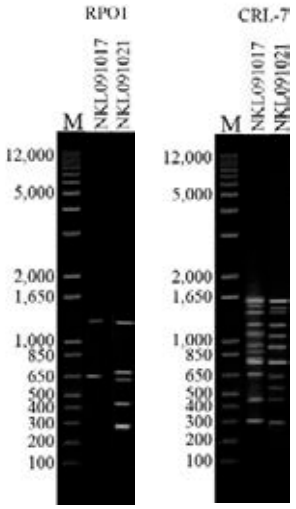


Figure C.24 The same strain: NKL091017=NKL091021.

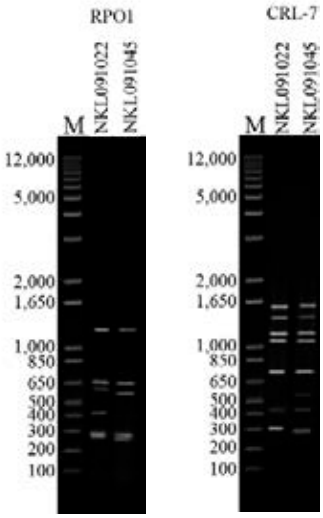


Figure C.25 The same strain: NKL091022=NKL091045.

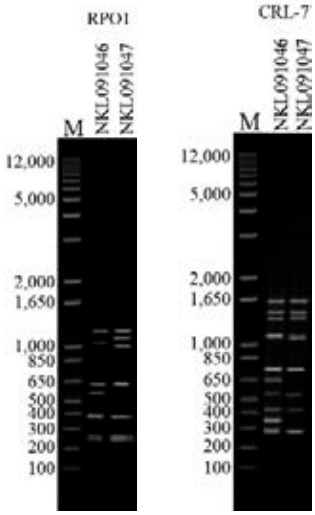


Figure C.26 The same strain: NKL091046=NKL091047.

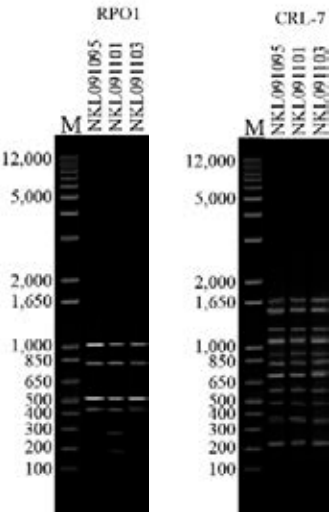


Figure C.27 The same strain: NKL091095=NKL091101=NKL091103.

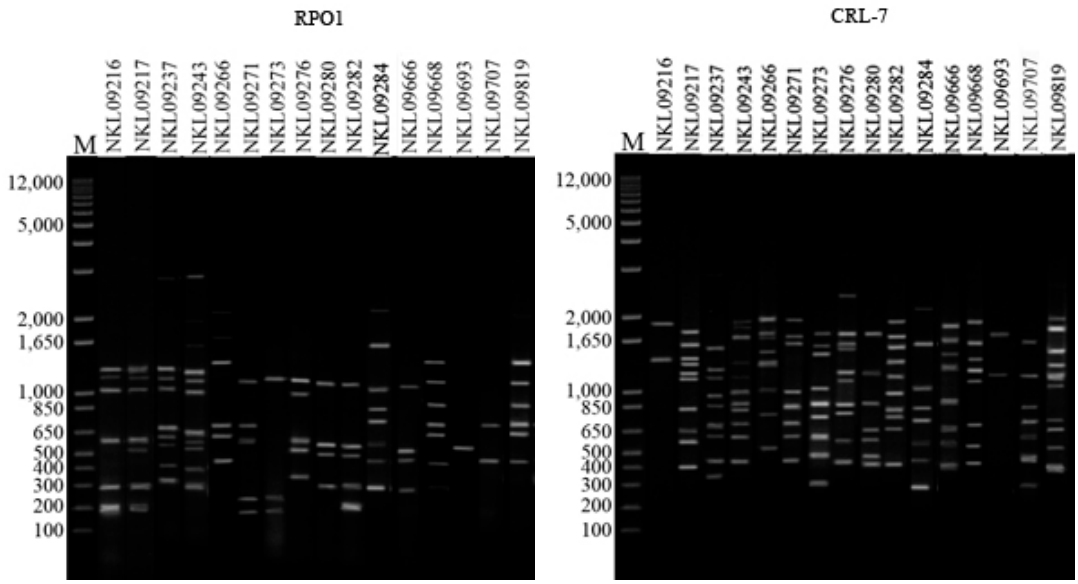


Figure C.28 Bacterial isolates with different RAPD-PCR fingerprints were different strains.

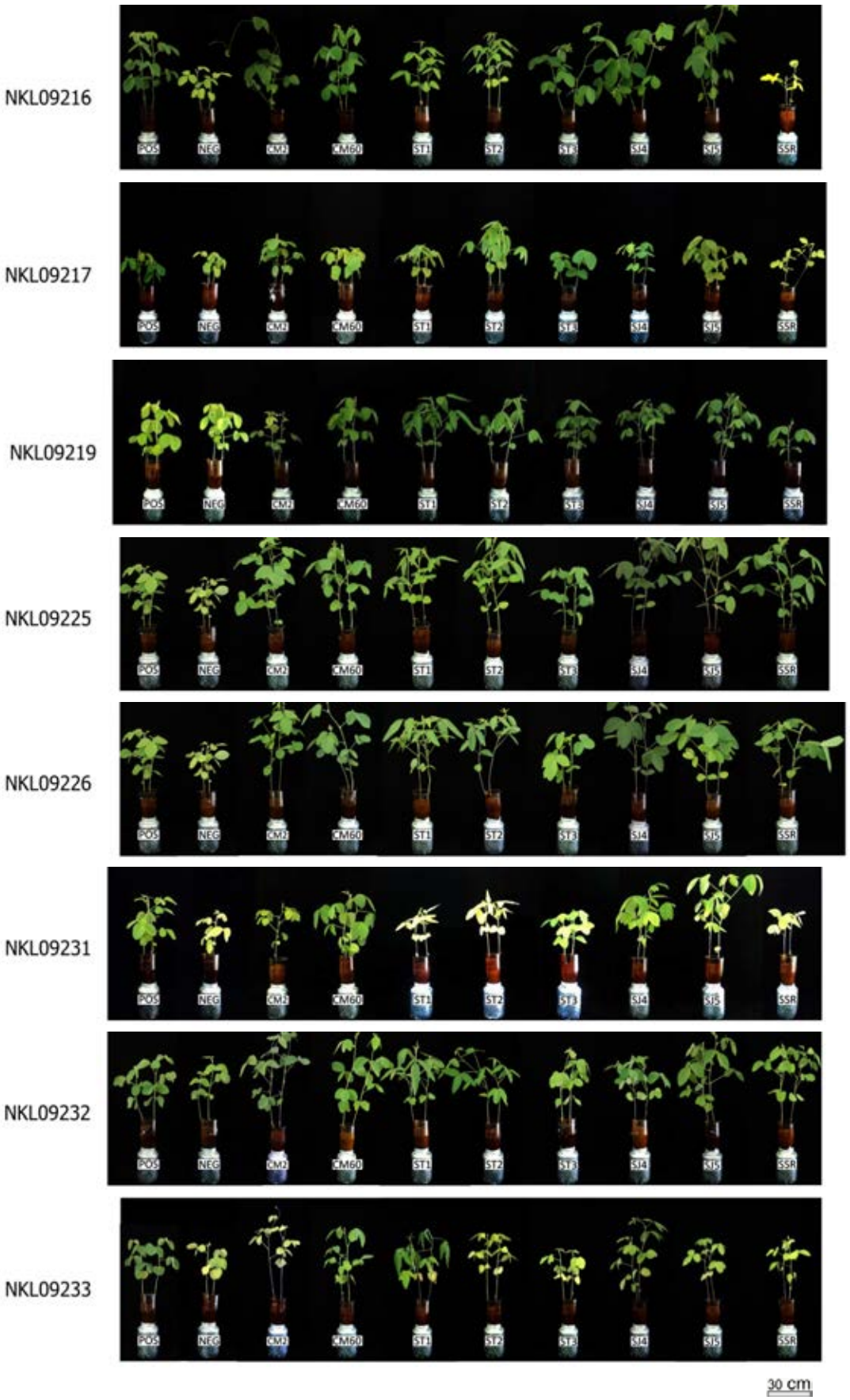
APPENDIX D

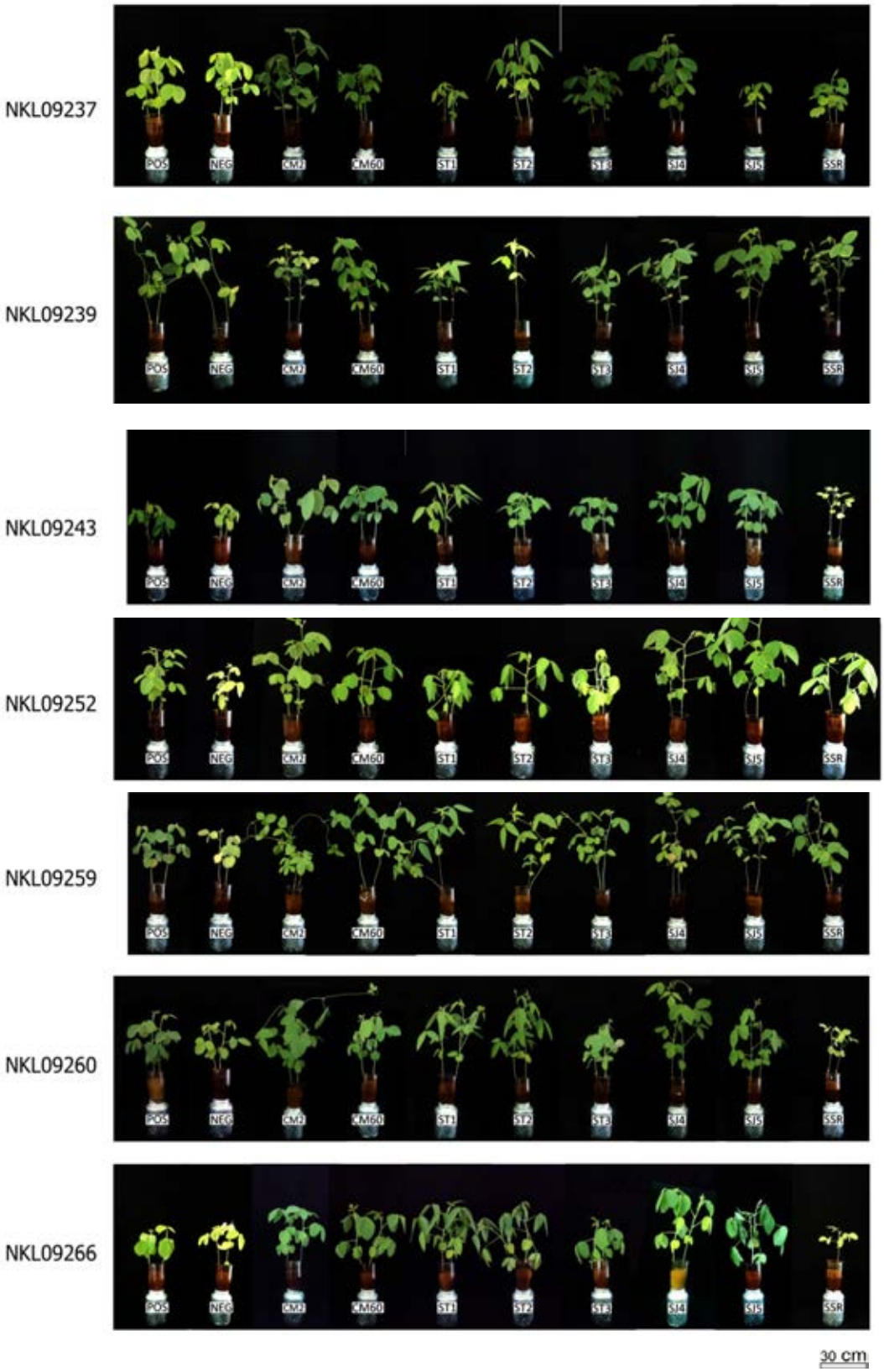
PRIMER SEQUNCES

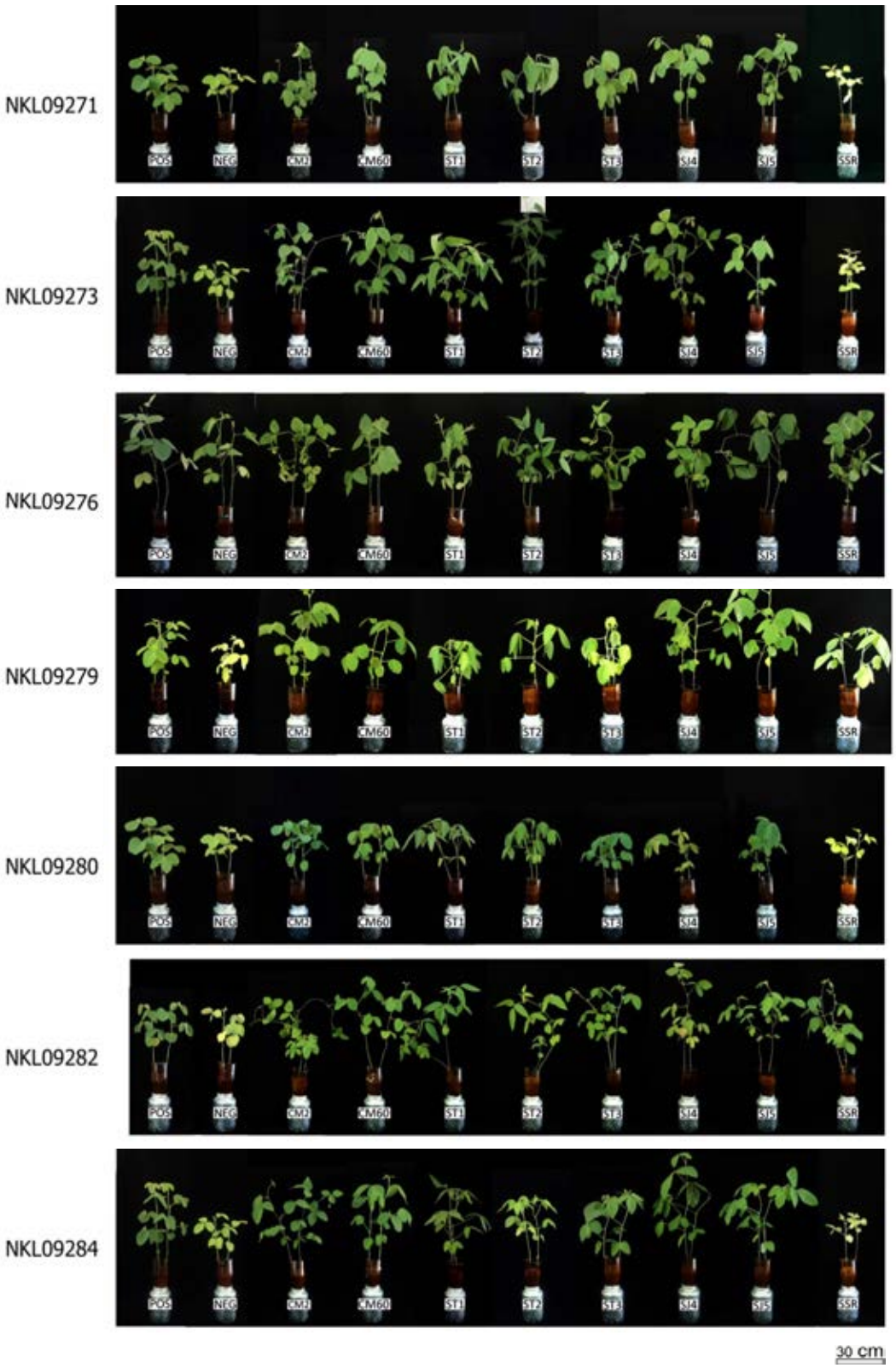
Primers	5'-3' sequences	PCR products (bp)	%GC	T_m (°C)
dnaK				
Forward primer	5' GTGTTGGTCTTGGCCTTGAT 3' (20 bases)	372	50.00	58.38
Reverse primer	5' CGGCATCGTCAACGTCT 3' (17 bases)		58.82	57.36
glnII				
Forward primer	5' GATGCCGTACTTCTCGGTCA 3' (20 bases)	407	55.00	59.55
Reverse primer	5' TGCTGGTGATGTGCGAAGTC 3' (20 bases)		55.00	60.95
glnII (NKL09273)				
Forward primer	5' GCCATCCACATTTCGTCAGC 3' (20 bases)	509	55.00	59.90
Reverse primer	5' GAATTCGCGTCGTTCCCGA 3' (19 bases)		57.89	60.80
nifH				
Forward primer	5' AGCCACCGCAAACAACGTCG 3' (20 bases)	363	60.0	62.9
Reverse primer	5' ATCGGCAAGTCCACCACTTC 3' (20 bases)		55.0	60.8
recA				
Forward primer	5' CACCGAATCGACCACCAGAA 3' (20 bases)	260	55.00	60.04
Reverse primer	5' GCATCGTCGAGATCTACGGG 3' (20 bases)		60.00	60.11

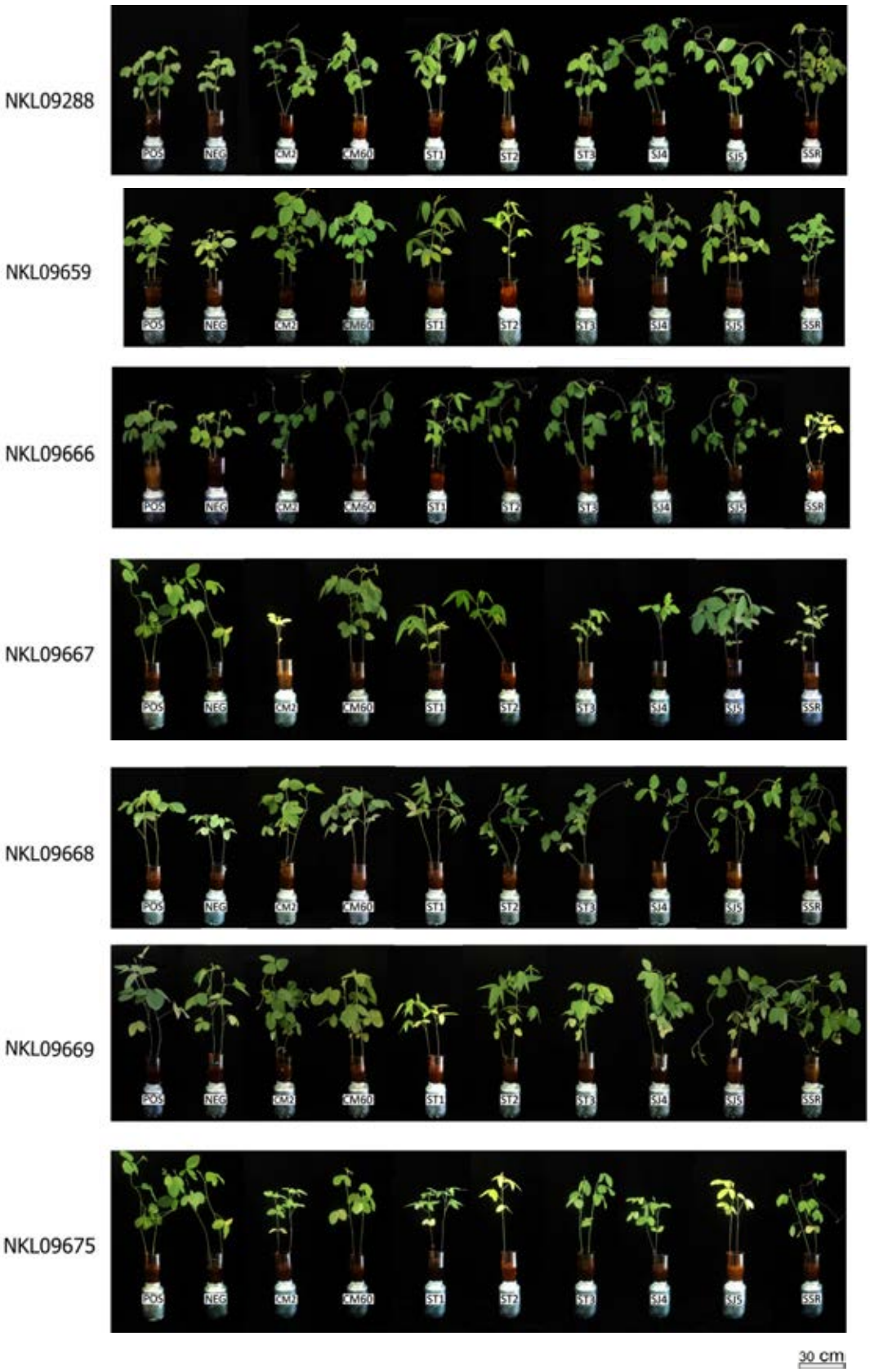
APPENDIX E

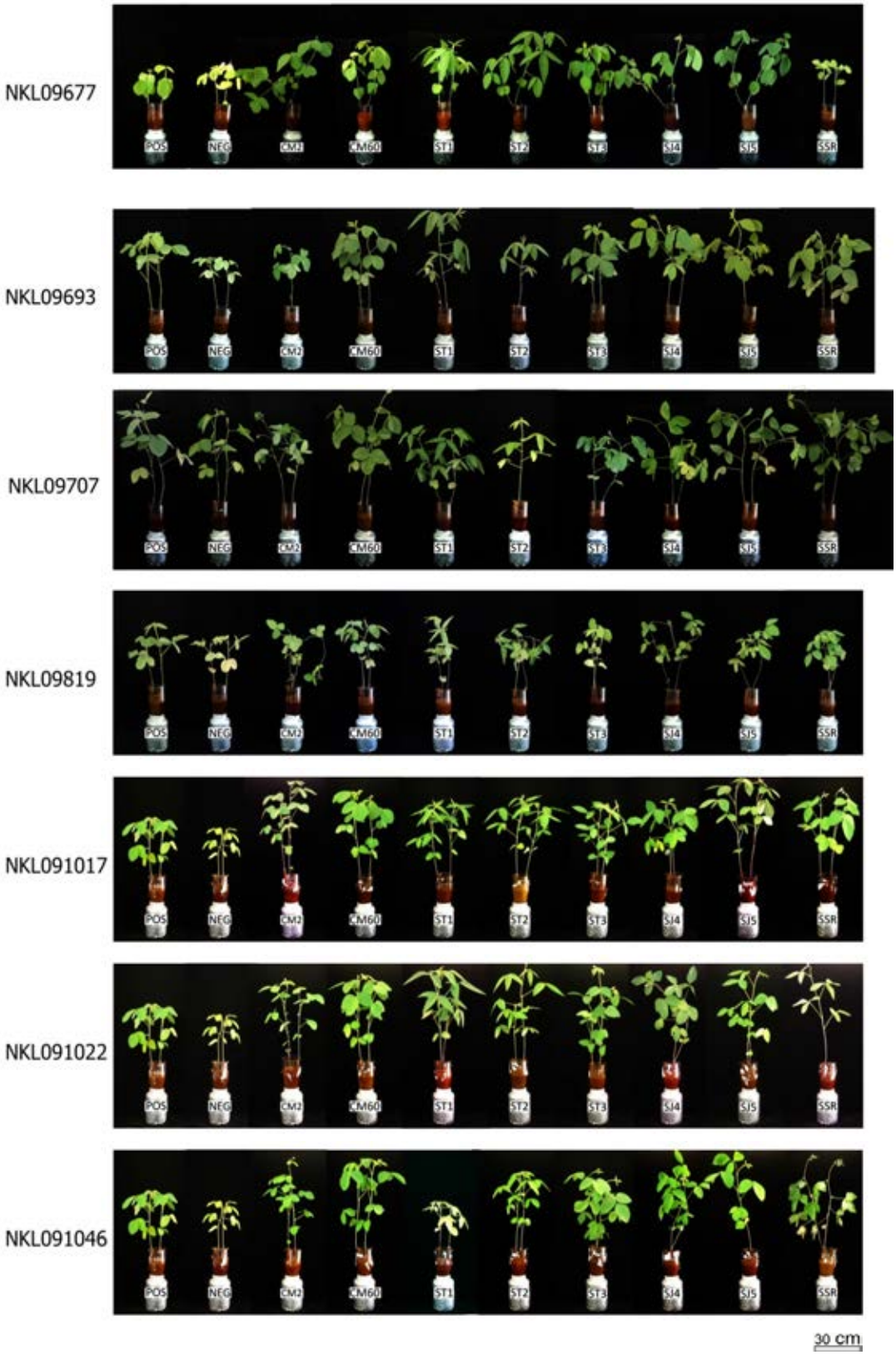
AUTHENTICATION TESTS

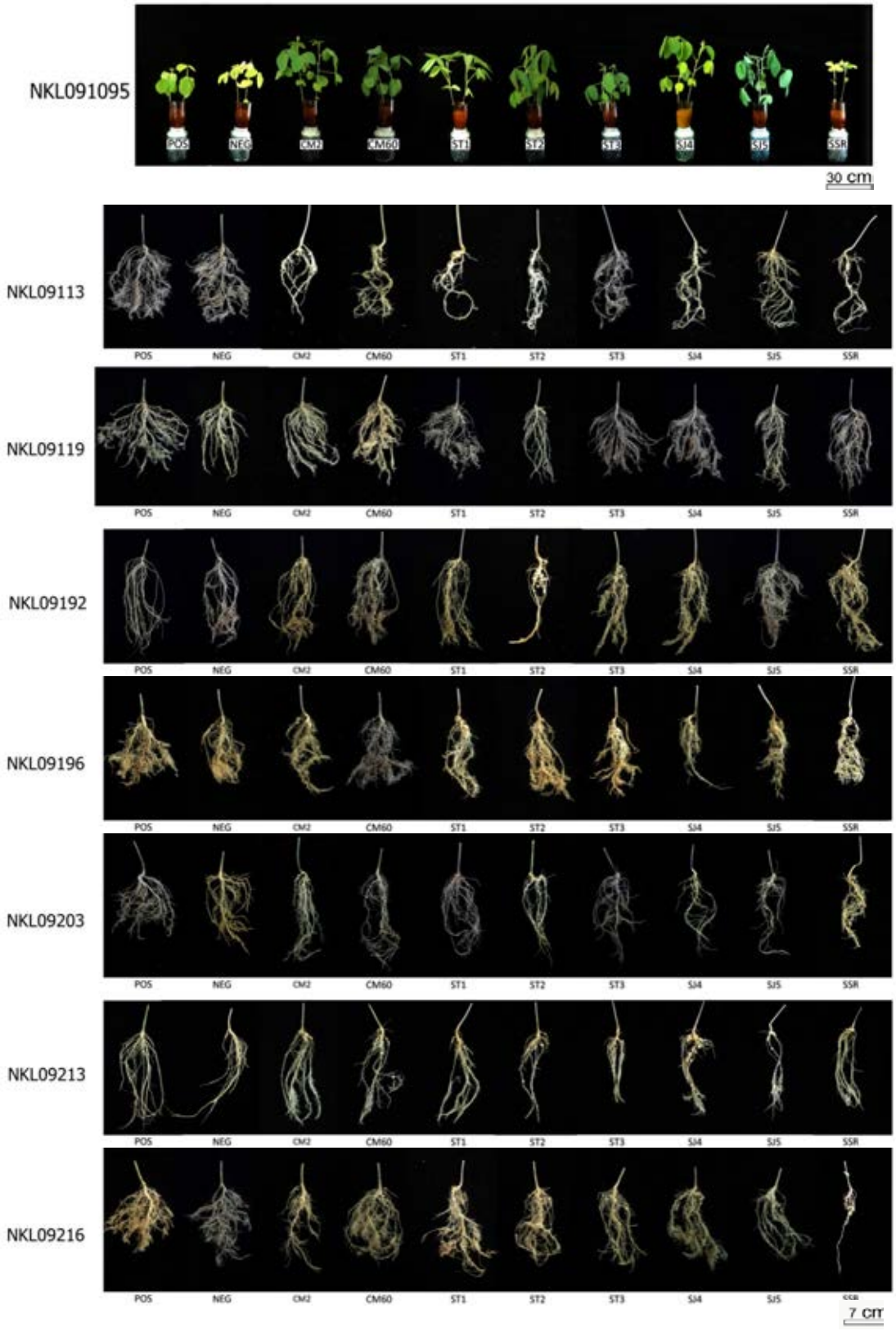

NKL09216
NKL09217
NKL09219
NKL09225
NKL09226
NKL09231
NKL09232
NKL09233
POS
NEG
CM2
CM60
ST1
ST2
ST3
ST4
ST5
SSR
30 cm

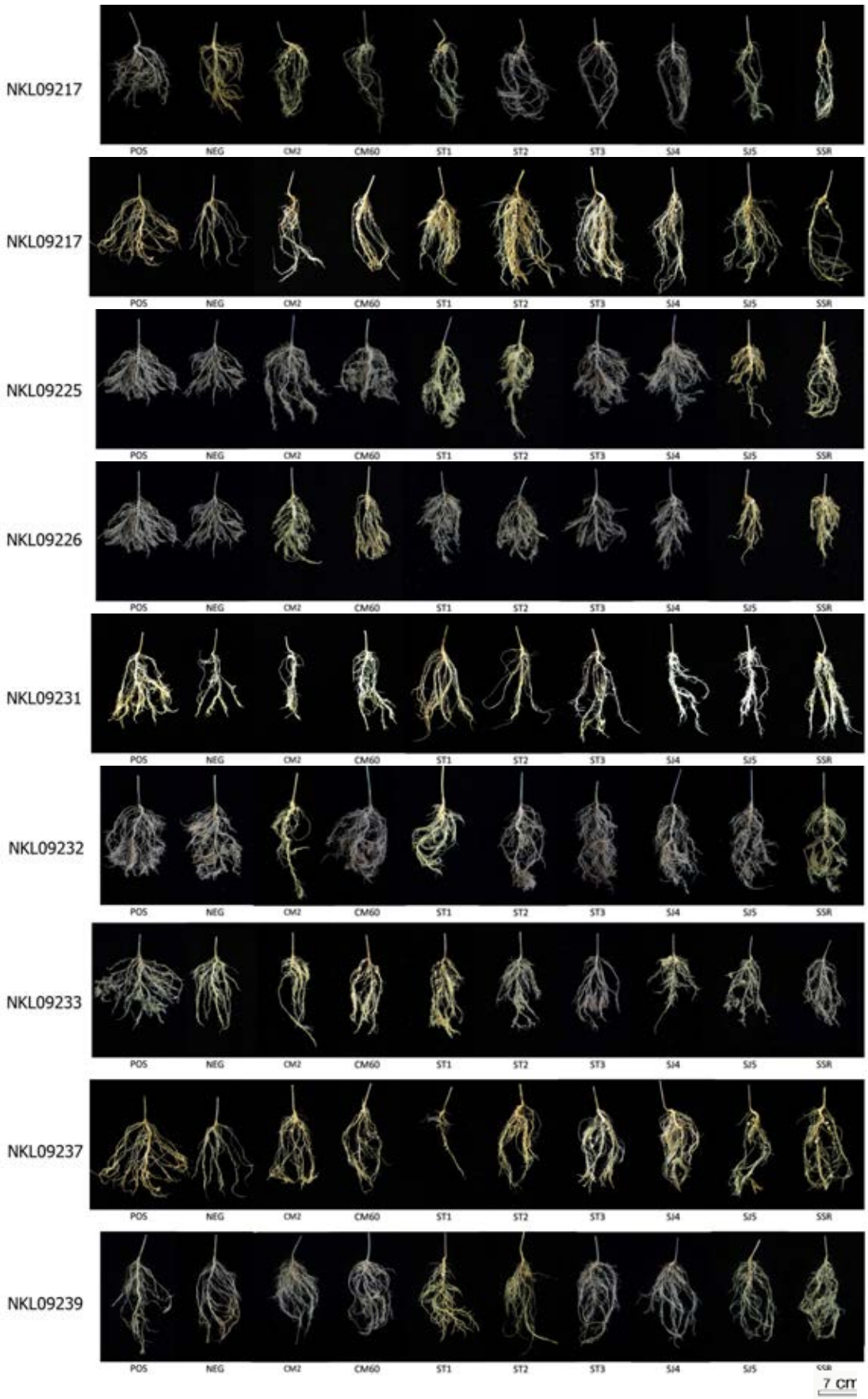

NKL09237
NKL09239
NKL09243
NKL09252
NKL09259
NKL09260
NKL09266
POS
NEG
CM2
CM60
ST1
ST2
ST3
SJ4
SJ5
SSR
30 cm

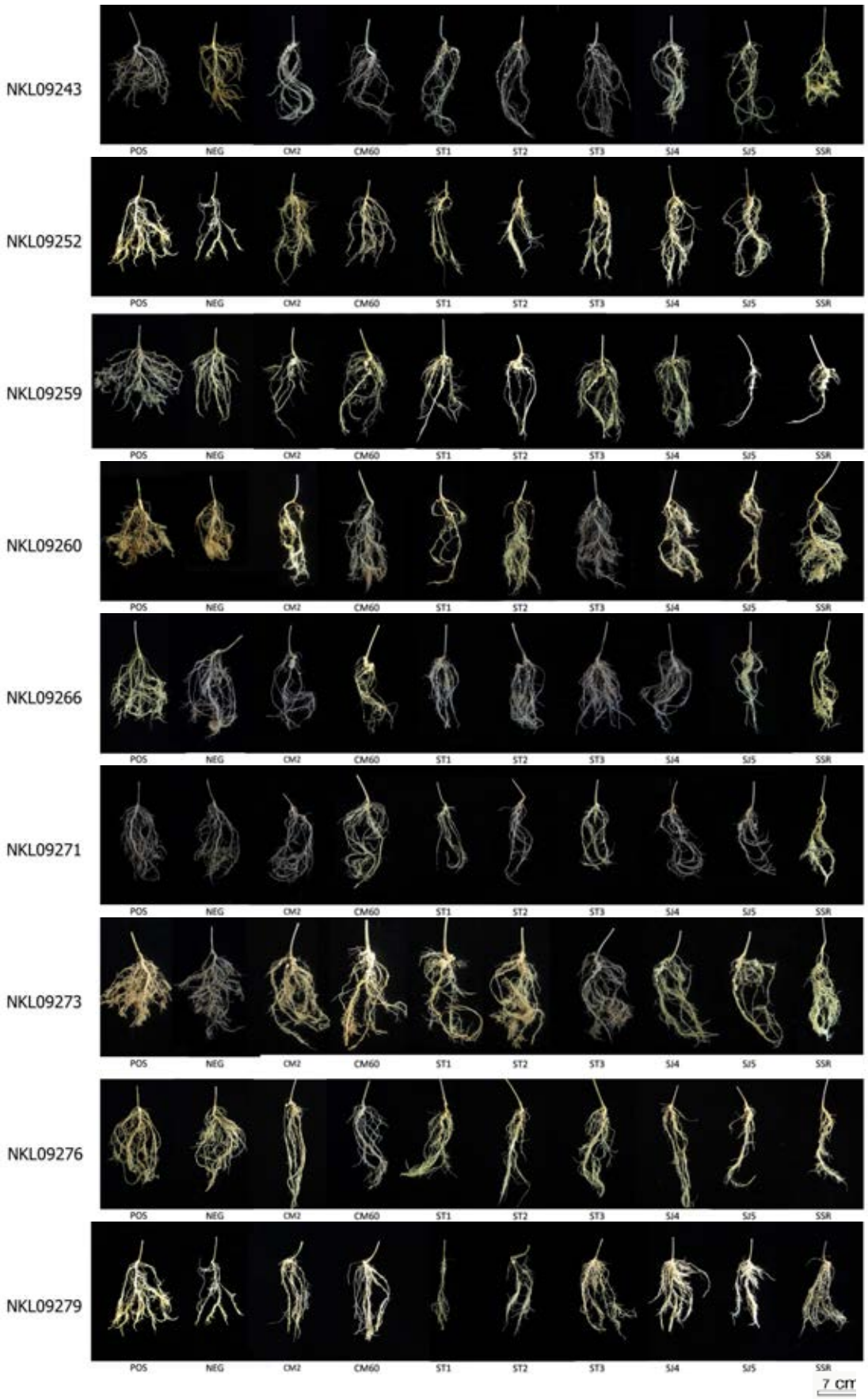

NKL09271
NKL09273
NKL09276
NKL09279
NKL09280
NKL09282
NKL09284
POS
NEG
CM3
CM60
ST1
ST2
ST3
SJ4
SJ5
SSR
30 cm

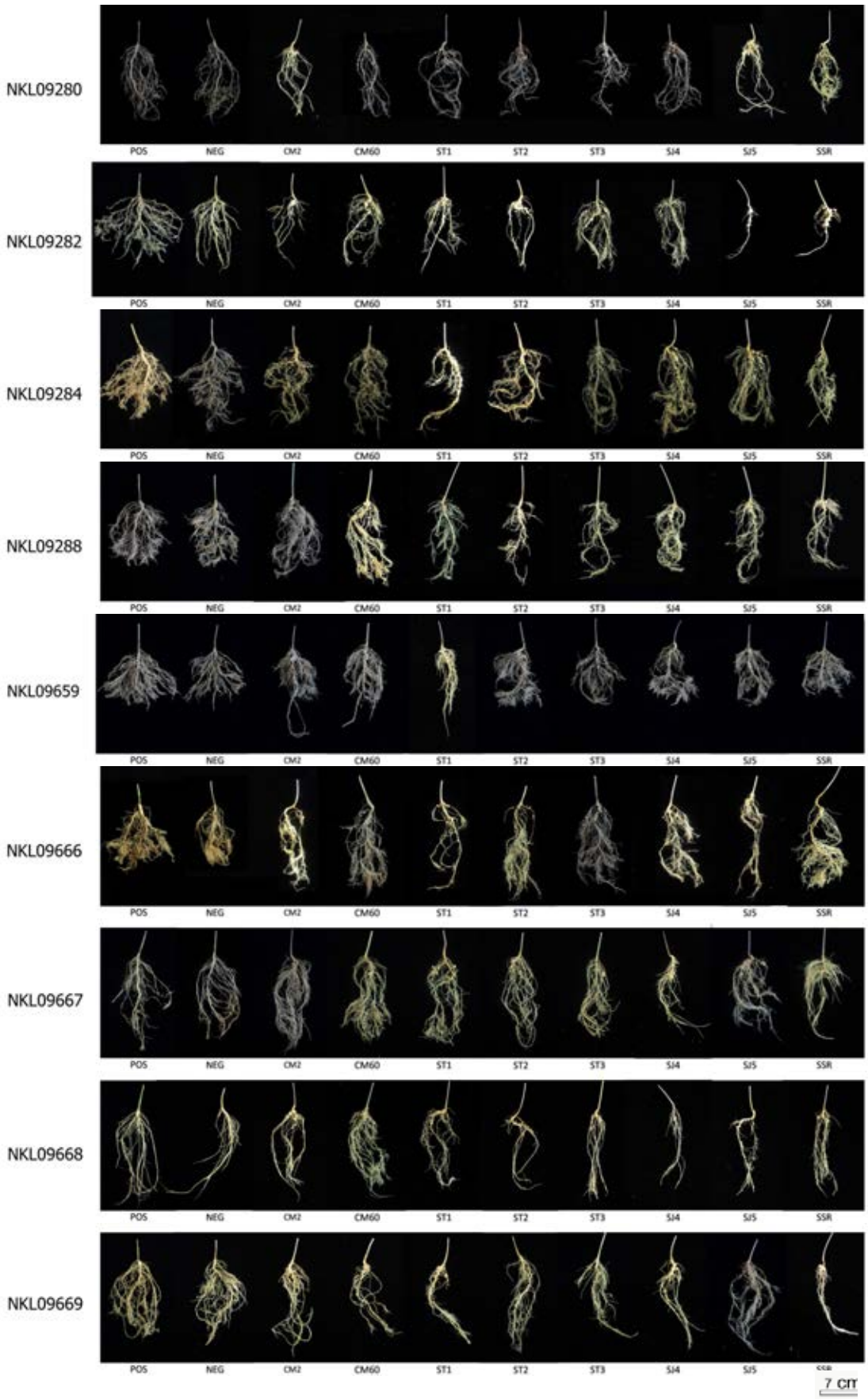

NKL09288
NKL09659
NKL09666
NKL09667
NKL09668
NKL09669
NKL09675
POS
NEG
CM2
CM60
ST1
ST2
ST3
SJ4
SJ5
SSR
30 cm

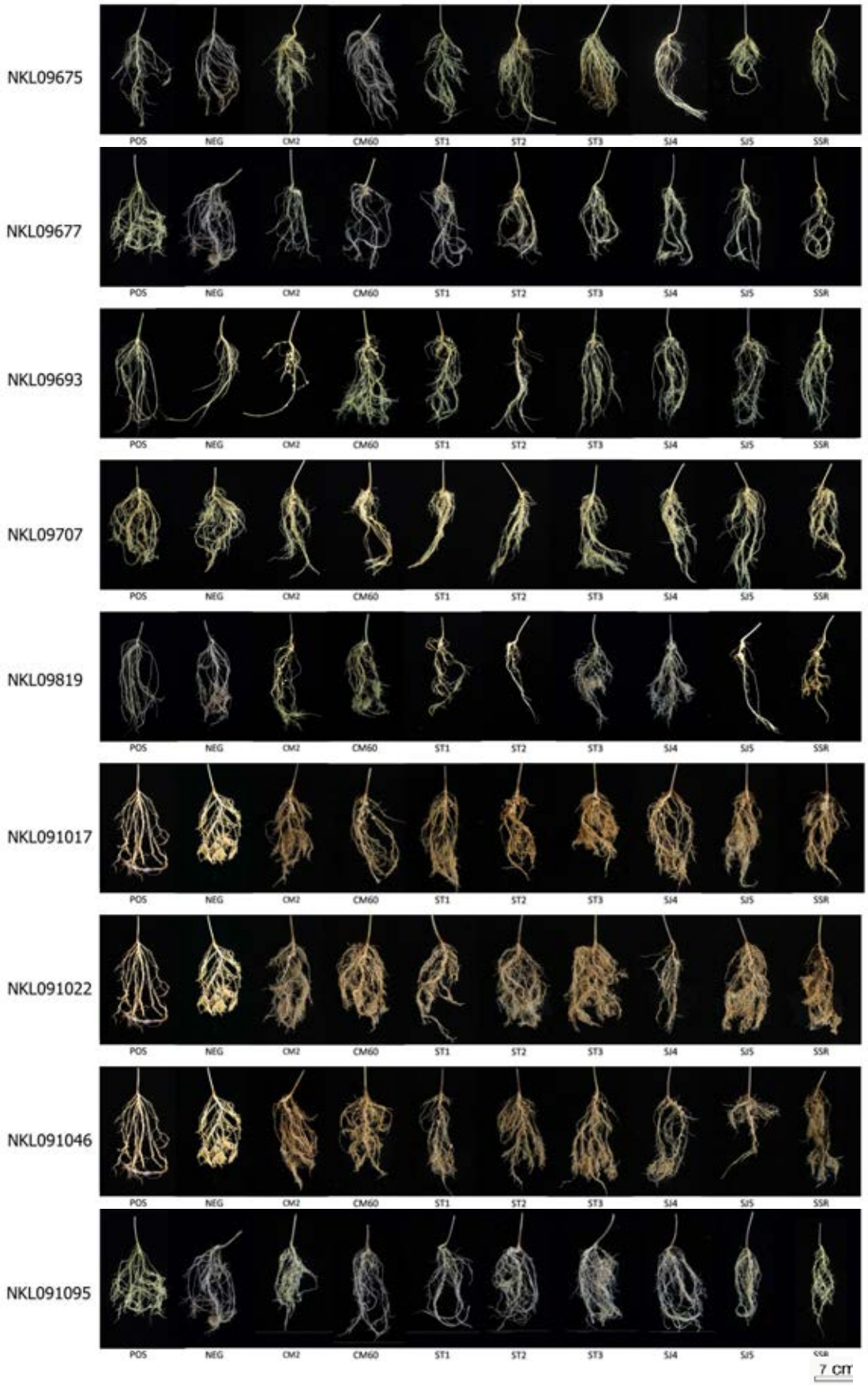

NKL09677
NKL09693
NKL09707
NKL09819
NKL091017
NKL091022
NKL091046
POS
NEG
CM2
CM60
ST1
ST2
ST3
SJ4
SJ5
SSR
30 cm


NKL091095
POS
NEG
CM2
CM60
ST1
ST2
ST3
SJ4
SJ5
SSR
30 cm
NKL09113
NKL09119
NKL09192
NKL09196
NKL09203
NKL09213
NKL09216
7 cm


NKL09217
NKL09217
NKL09225
NKL09226
NKL09231
NKL09232
NKL09233
NKL09237
NKL09239
POS
NEG
CM2
CM60
ST1
ST2
ST3
SJ4
SJ5
SSR
7 cm


NKL09243
NKL09252
NKL09259
NKL09260
NKL09266
NKL09271
NKL09273
NKL09276
NKL09279
POS
NEG
CM2
CM60
ST1
ST2
ST3
SJ4
SJ5
SSR
7 cm


NKL09280
NKL09282
NKL09284
NKL09288
NKL09659
NKL09666
NKL09667
NKL09668
NKL09669
POS
NEG
CM2
CM60
ST1
ST2
ST3
SJ4
SJ5
SSR
7 cm


NKL09675
NKL09677
NKL09693
NKL09707
NKL09819
NKL091017
NKL091022
NKL091046
NKL091095
POS
NEG
CM2
CM60
ST1
ST2
ST3
SJ4
SJ5
SSR
7 cm

Results of Total Nitrogen by Kjeldahl Method				
Strains	Determination	Total Nitrogen g /100g	Average	SD
NKL09113	1	0.73	0.54	0.269
	2	0.35		
NKL09119	1	0.28	0.35	0.092
	2	0.41		
NKL09192	1	0.54	0.61	0.099
	2	0.68		
NKL09196	1	0.44	0.45	0.007
	2	0.45		
NKL09203	1	0.45	0.52	0.092
	2	0.58		
NKL09213	1	0.69	0.60	0.127
	2	0.51		
NKL09216*	1	0.67	0.61	0.092
	2	0.54		
NKL09217	1	0.38	0.43	0.064
	2	0.47		
NKL09219	1	0.86	0.56	0.421
	2	0.26		
NKL09225	1	0.38	0.36	0.035
	2	0.33		
NKL09226	1	0.47	0.43	0.057
	2	0.39		
NKL09231*	1	0.76	0.84	0.114
	2	0.92		
NKL09232	1	0.50	0.425	0.106
	2	0.35		
NKL09233	1	0.14	0.190	0.071
	2	0.24		
NKL09237	1	0.86	0.51	0.502
	2	0.15		
NKL09239	1	0.41	0.42	0.007
	2	0.42		

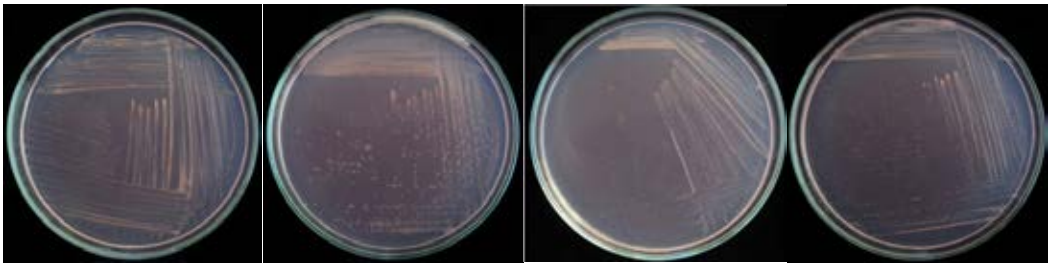
Results of Total Nitrogen by Kjeldahl Method				
Strains	Determination	Total Nitrogen g /100g	Average	SD
NKL09243	1	0.51	0.52	0.014
	2	0.53		
NKL09252	1	0.57	0.51	0.080
	2	0.45		
NKL09259	1	0.31	0.36	0.064
	2	0.40		
NKL09260	1	0.40	0.47	0.092
	2	0.53		
NKL09266	1	0.41	0.49	0.106
	2	0.56		
NKL09271	1	0.69	0.52	0.247
	2	0.34		
NKL09273*	1	0.61	0.69	0.106
	2	0.76		
NKL09276	1	0.66	0.65	0.021
	2	0.63		
NKL09279	1	0.58	0.75	0.240
	2	0.92		
NKL09280	1	0.45	0.45	0.000
	2	0.45		
NKL09282	1	0.57	0.46	0.156
	2	0.35		
NKL09284	1	0.74	0.65	0.134
	2	0.55		
NKL09288	1	0.49	0.44	0.071
	2	0.39		
NKL09659	1	0.39	0.39	0.007
	2	0.40		
NKL09666*	1	0.73	0.73	0.000
	2	0.73		
NKL09667	1	0.42	0.41	0.021
	2	0.39		

Results of Total Nitrogen by Kjeldahl Method				
Strains	Determination	Total Nitrogen g /100g	Average	SD
NKL09668	1	1.02	0.85	0.247
	2	0.67		
NKL09669	1	0.69	0.60	0.127
	2	0.51		
NKL09675	1	0.42	0.41	0.007
	2	0.41		
NKL09677	1	0.58	0.75	0.240
	2	0.92		
NKL09693*	1	0.89	0.84	0.071
	2	0.79		
NKL09707	1	0.59	0.50	0.134
	2	0.40		
NKL09819	1	0.51	0.50	0.014
	2	0.49		
NKL091017	1	0.27	0.38	0.156
	2	0.49		
NKL091022	1	0.40	0.44	0.057
	2	0.48		
NKL091046	1	0.45	0.41	0.057
	2	0.37		
NKL091095	1	0.42	0.55	0.177
	2	0.67		

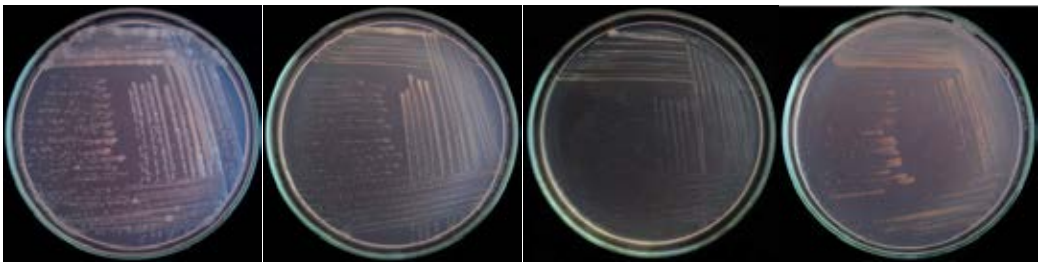
*Strains selected for identification by Multilocus sequence Analysis.

APPENDIX F

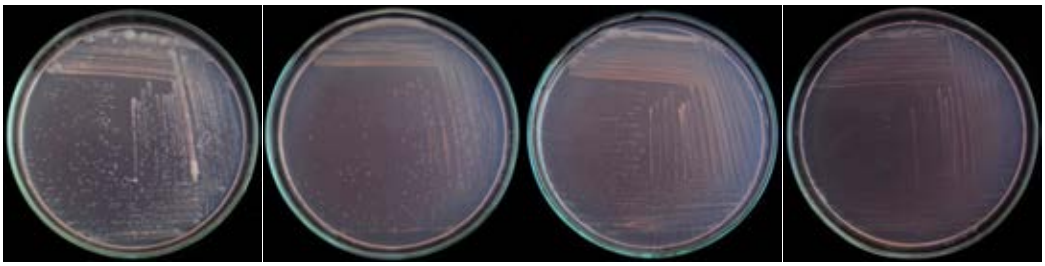
COLONY MORPHOLOGY AFTER 7-DAY INCUBATION AT 30°C



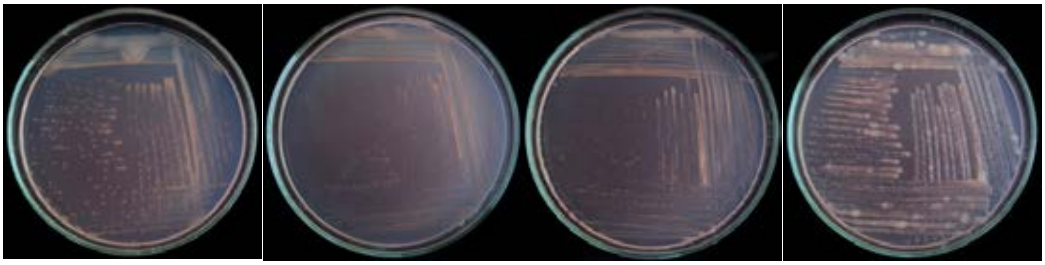
NKL09113 NKL09119 NKL09192 NKL09196



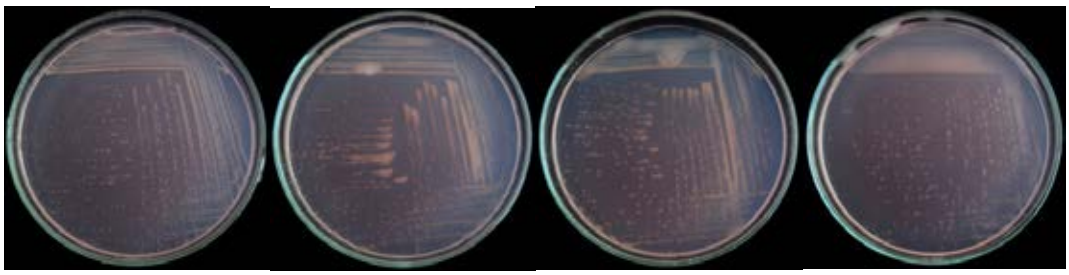
NKL09203 NKL09213 NKL09216* NKL09217



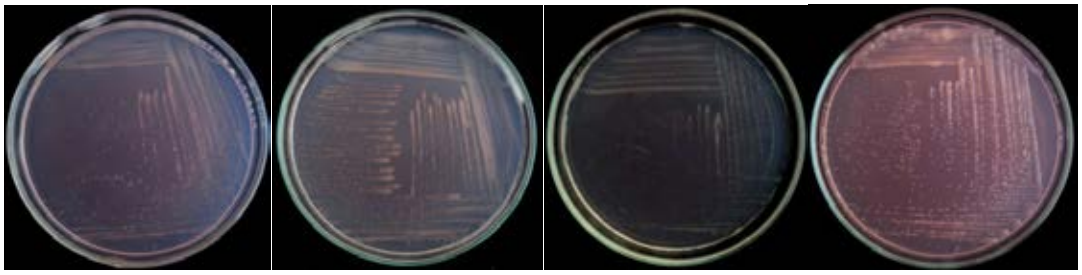
NKL09219 NKL09225 NKL09226 NKL09231*



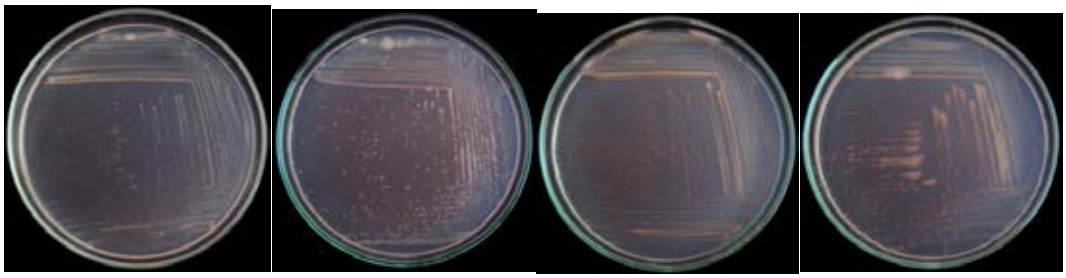
NKL09232 NKL09233 NKL09237 NKL09239



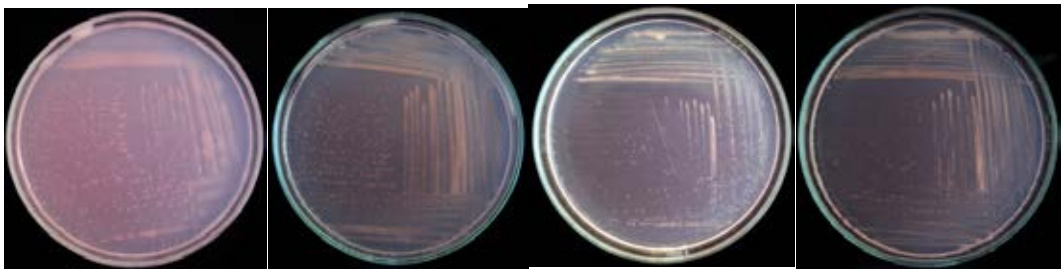
NKL09243 NKL09252 NKL09259 NKL09260



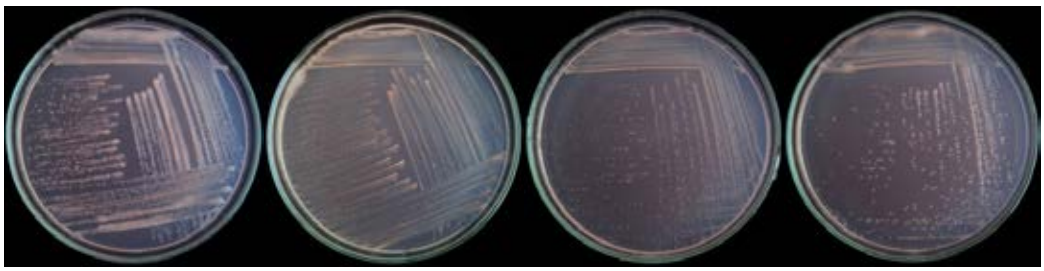
NKL09266 NKL09271 NKL09273* NKL09276



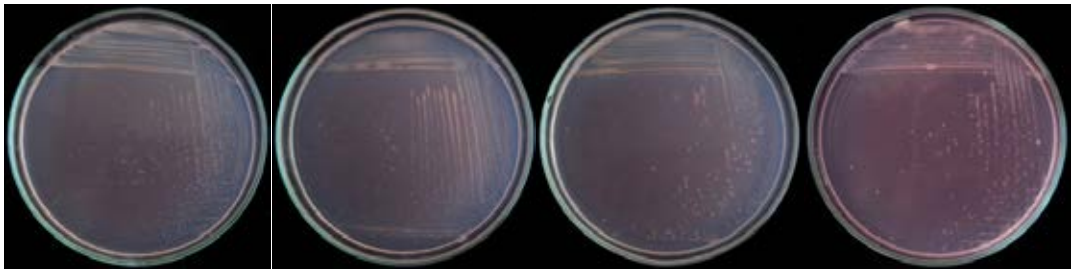
NKL09279 NKL09280 NKL09282 NKL09284



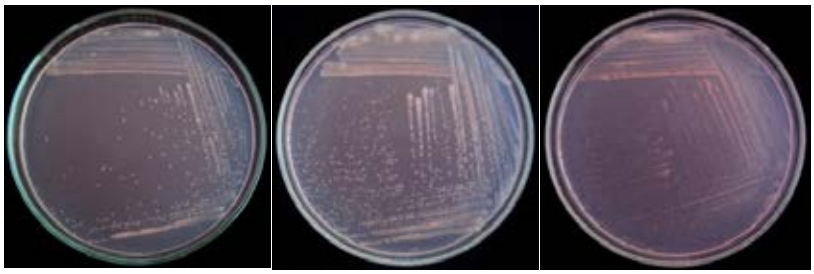
NKL09288 NKL09659 NKL09666* NKL09667



NKL09668 NKL09669 NKL09675 NKL09677



NKL09693* NKL09707 NKL09819 NKL091017

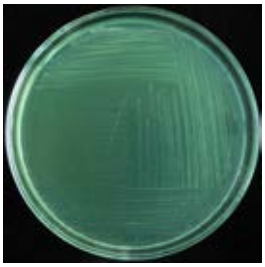


NKL091022 NKL091046 NKL091095

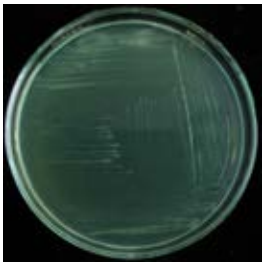
* Strains selected for identification by Multilocus Sequence Analysis.

APPENDIX G

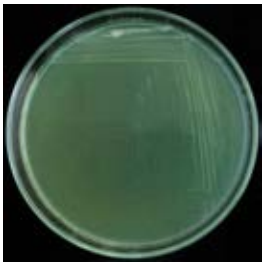
BROMOTHYMOL BLUE REACTIONS AFTER 5-DAY AND 10-DAY INCUBATION AT 30°C



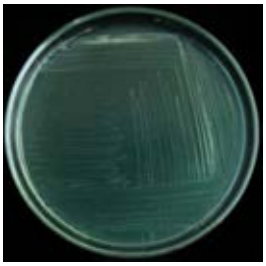
NKL09113



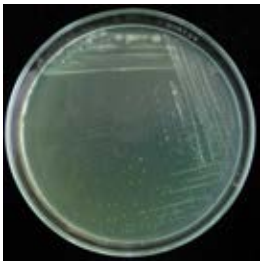
NKL09119



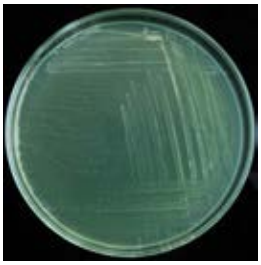
NKL09192



NKL09196



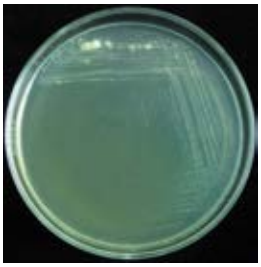
NKL09203



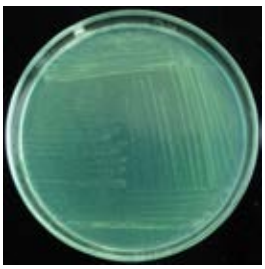
NKL09213*

NKL09216

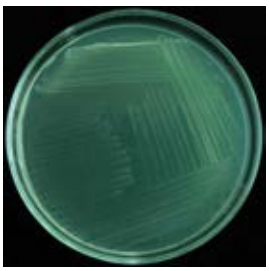
NKL09217



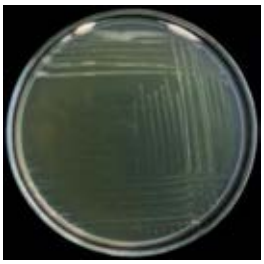
NKL09219



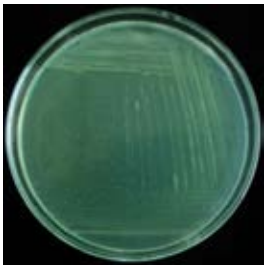
NKL09225



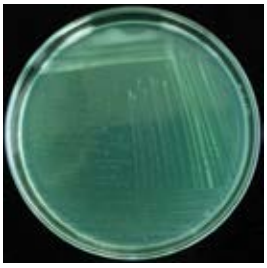
NKL09226



NKL09231*



NKL09232



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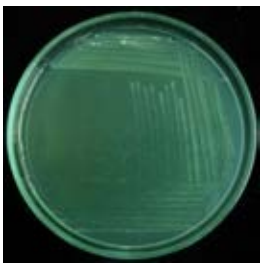
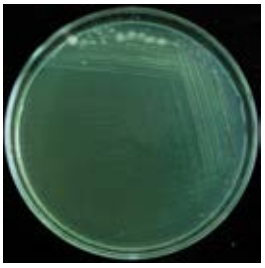


NKL09237



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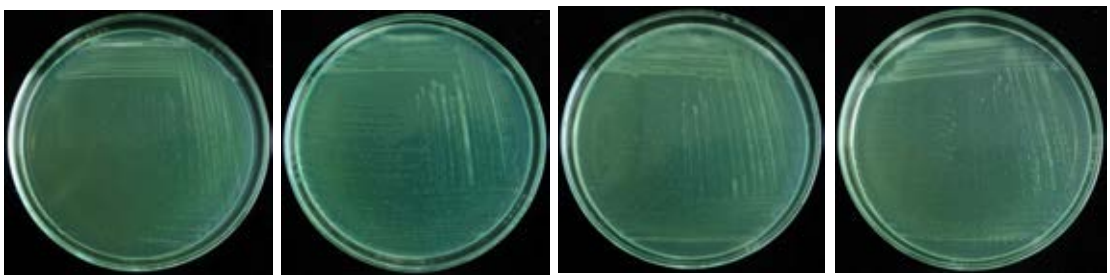
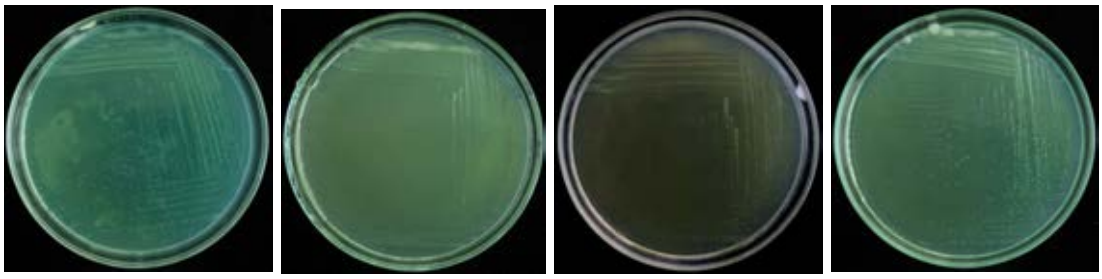
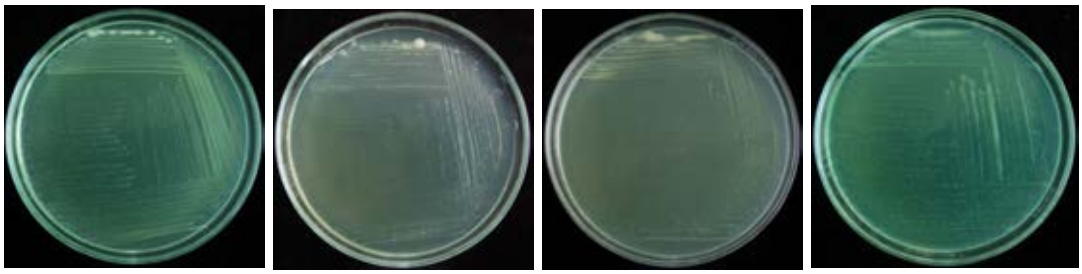


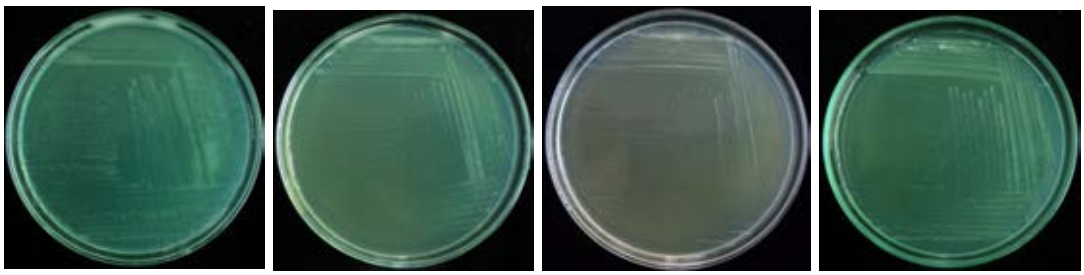
NKL09243 NKL09252 NKL09259 NKL09260



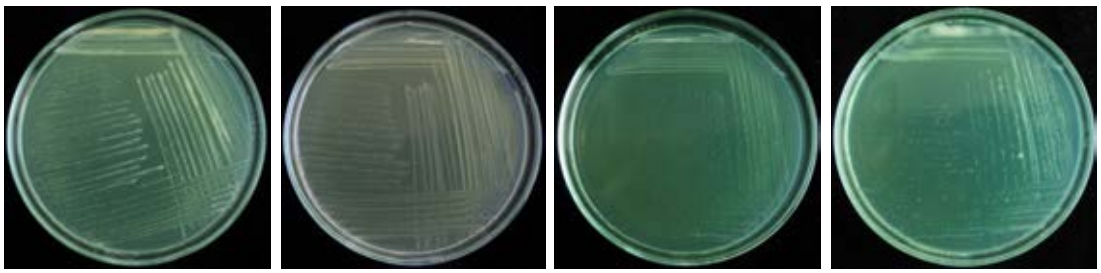
NKL09266 NKL09271 NKL09273* NKL09276



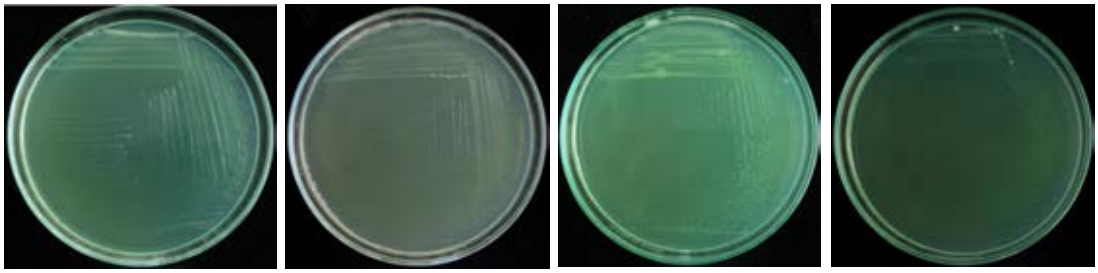
NKL09279 NKL09280 NKL09282 NKL09284



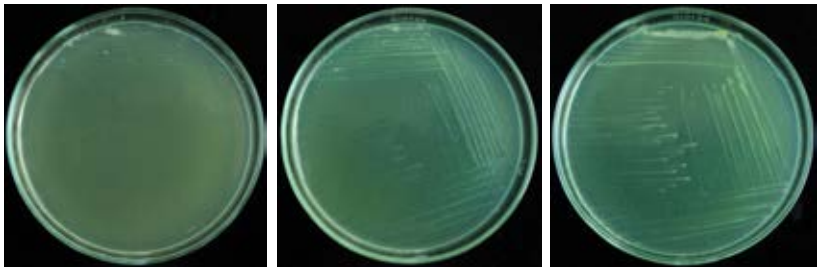
NKL09288 NKL09659 NKL09666* NKL09667



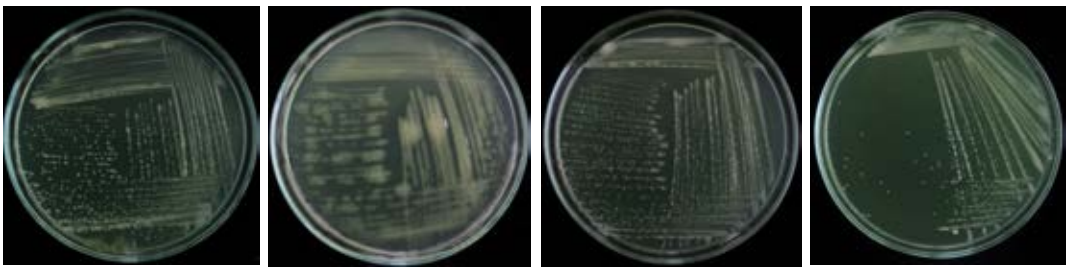
NKL09668 NKL09669 NKL09675 NKL09677



NKL09693* NKL09707 NKL09819 NKL091017

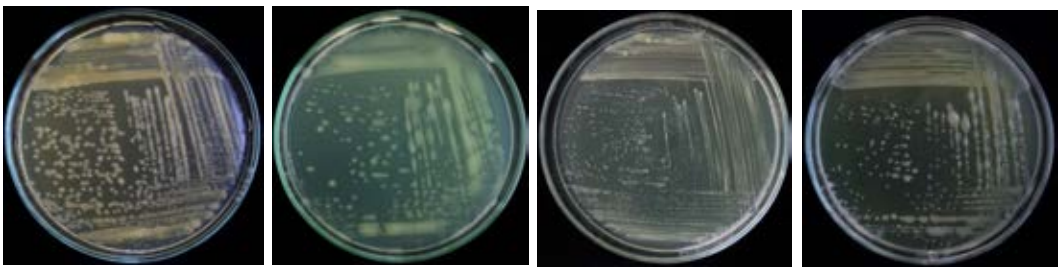


NKL091022 NKL091046 NKL091095

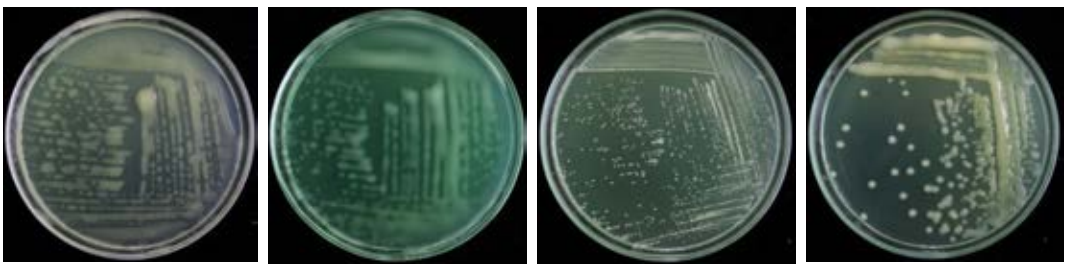


NKL09113 NKL09119 NKL09192 NKL09196

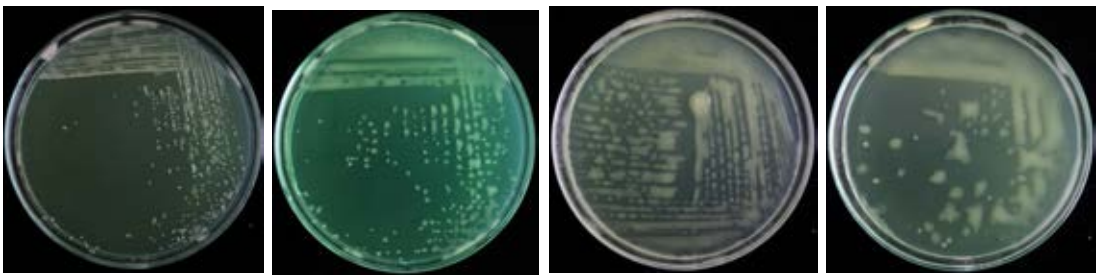
NKL09203 NKL09213 NKL09216* NKL09217



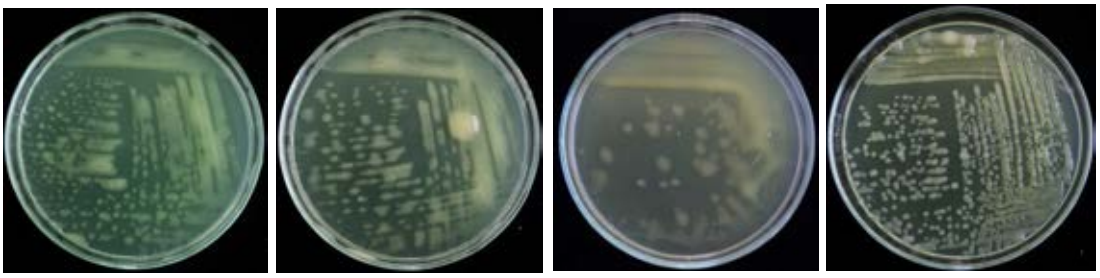
NKL09219 NKL09225 NKL09226 NKL09231*



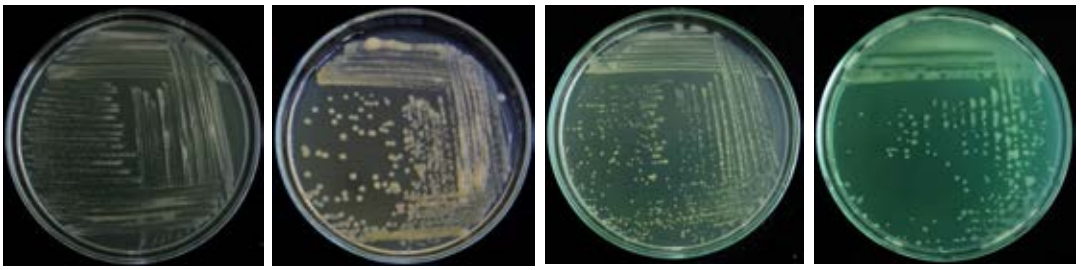
NKL09232 NKL09233 NKL09237 NKL09239



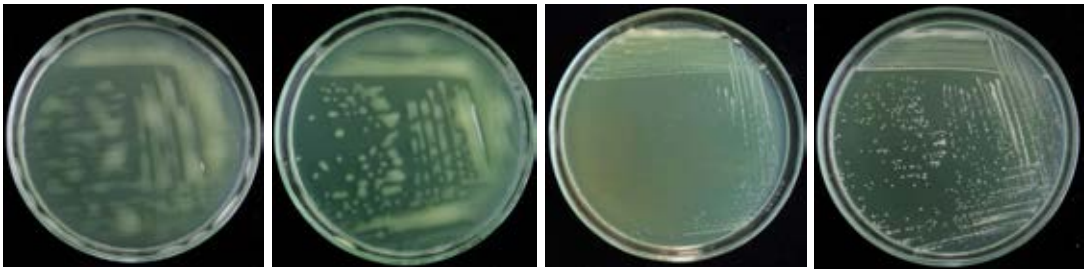
NKL09243 NKL09252 NKL09259 NKL09260



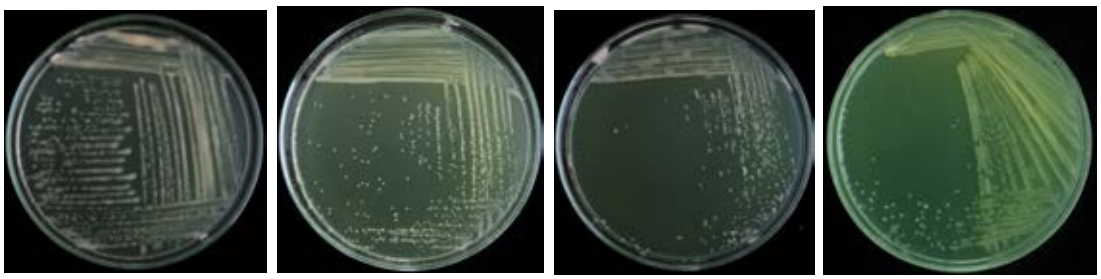
NKL09266 NKL09271 NKL09273* NKL09276



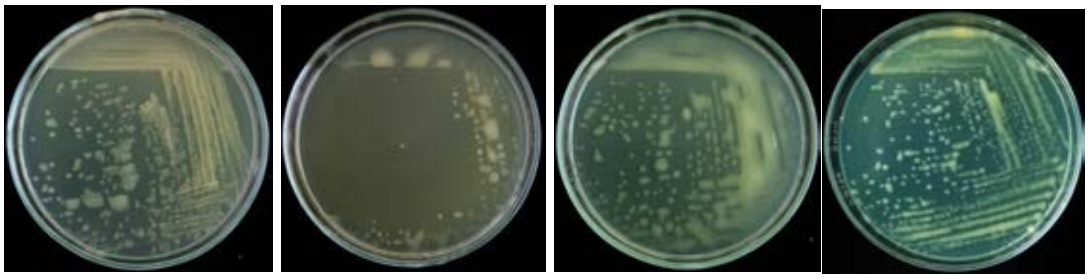
NKL09279 NKL09280 NKL09282 NKL09284



NKL09288 NKL09659 NKL09666* NKL09667



NKL09668 NKL09669 NKL09675 NKL09677



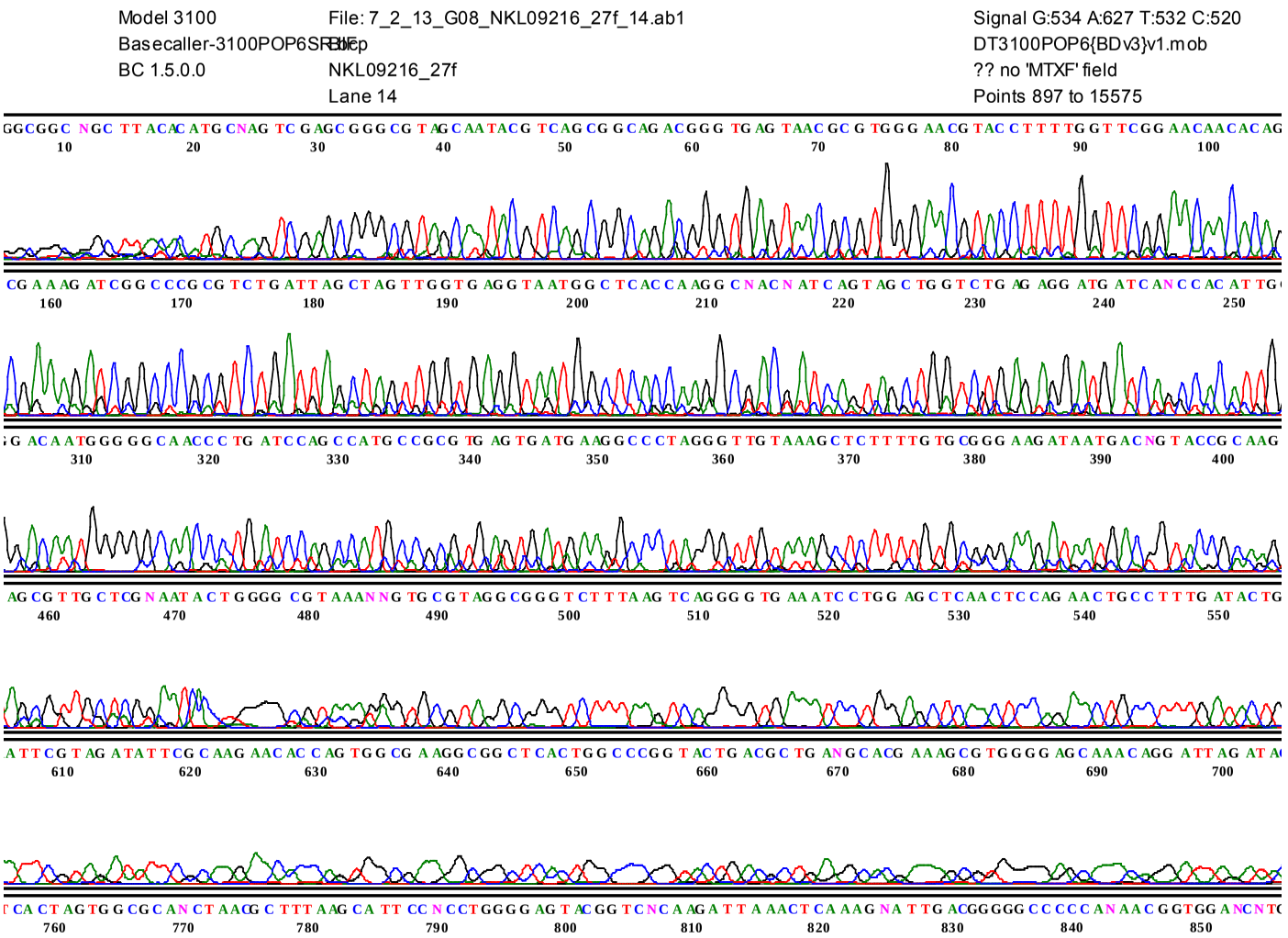
NKL09693* NKL09707 NKL09819 NKL091017

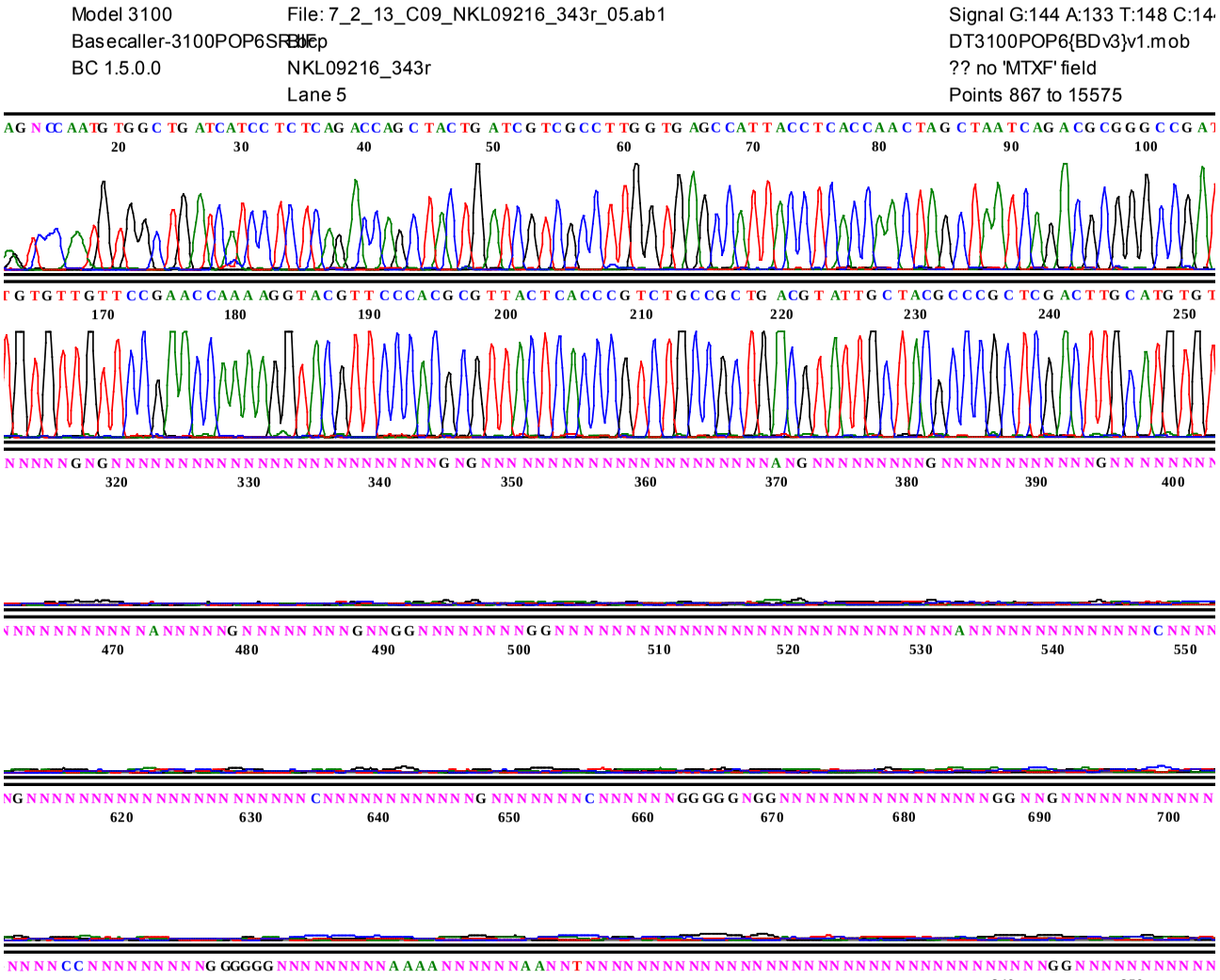
NKL091022 NKL091046 NKL091095

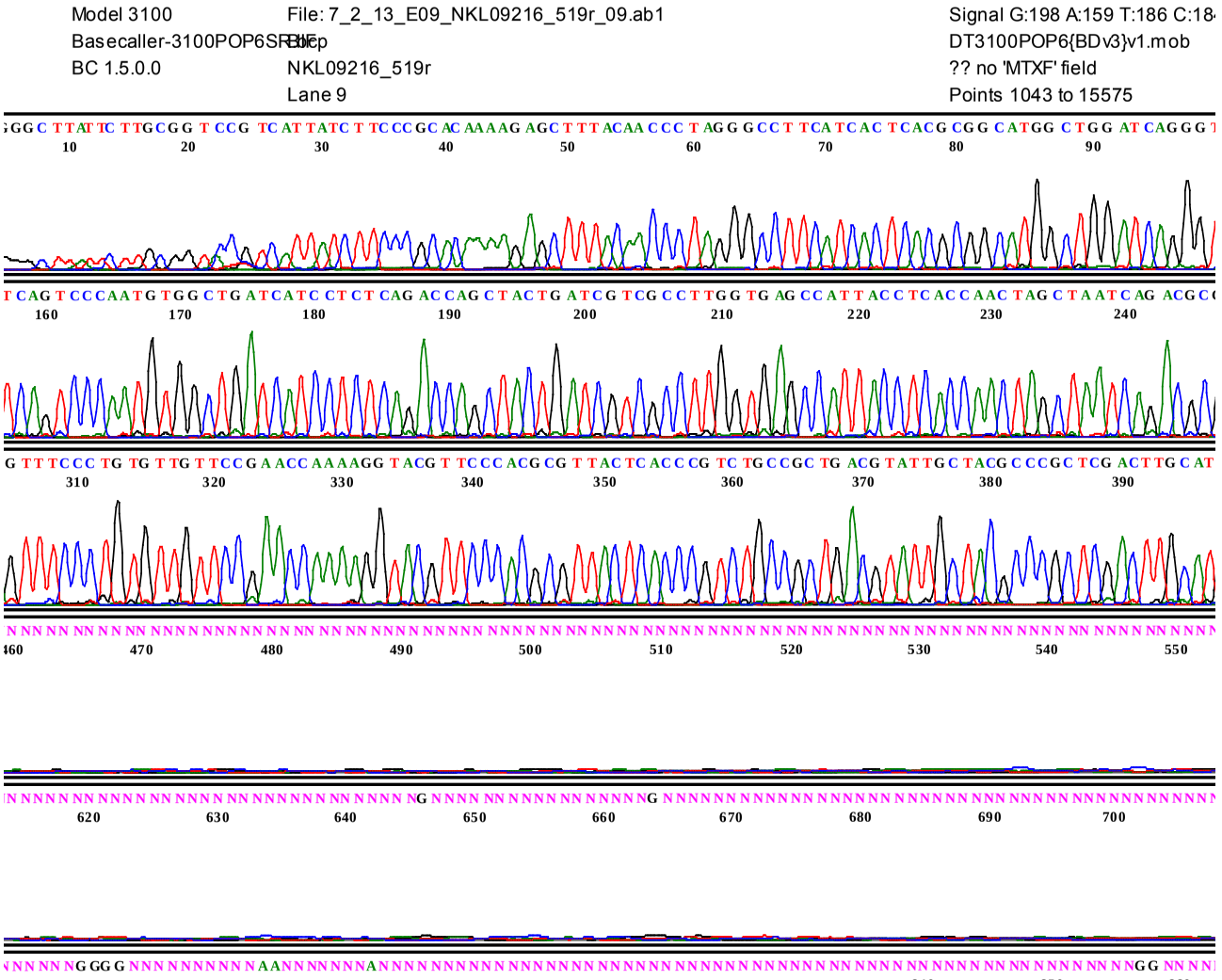
* Strains selected for identification by Multilocus Sequence Analysis.

APPENDIX H

SEQUENCING OF 16S rDNA, *dnaK*, *glnII*, *recA* AND *nifH*


Model 3100
Basecaller-3100POP6SRBC
BC 1.5.0.0
File: 7_2_13_G08_NKL09216_27f_14.ab1
Rerun
NKL09216_27f
Lane 14
Signal G:534 A:627 T:532 C:520
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 897 to 15575


Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 7_2_13_C09_NKL09216_343r_05.ab1
NKL09216_343r
Lane 5
Signal G:144 A:133 T:148 C:14
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 867 to 15575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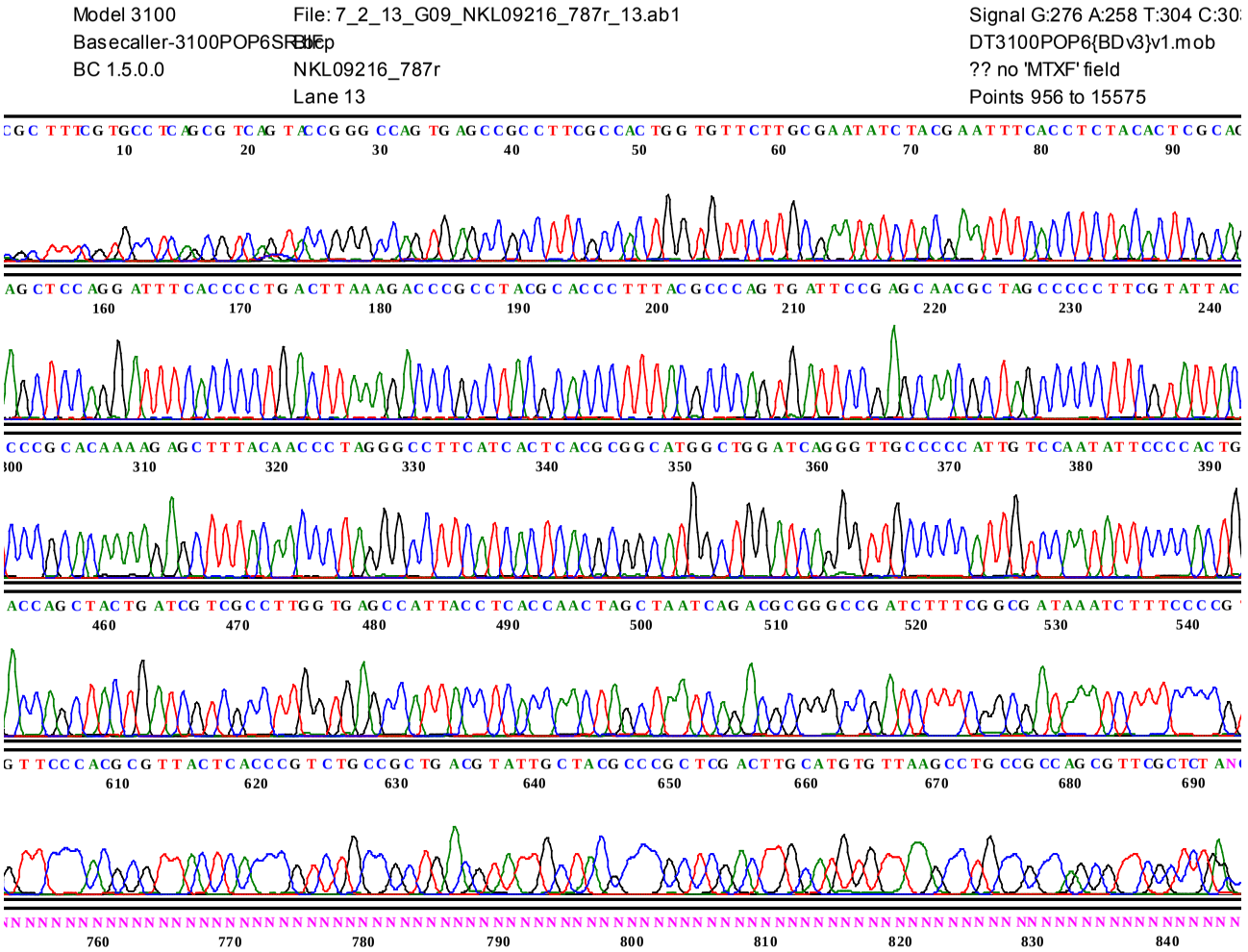
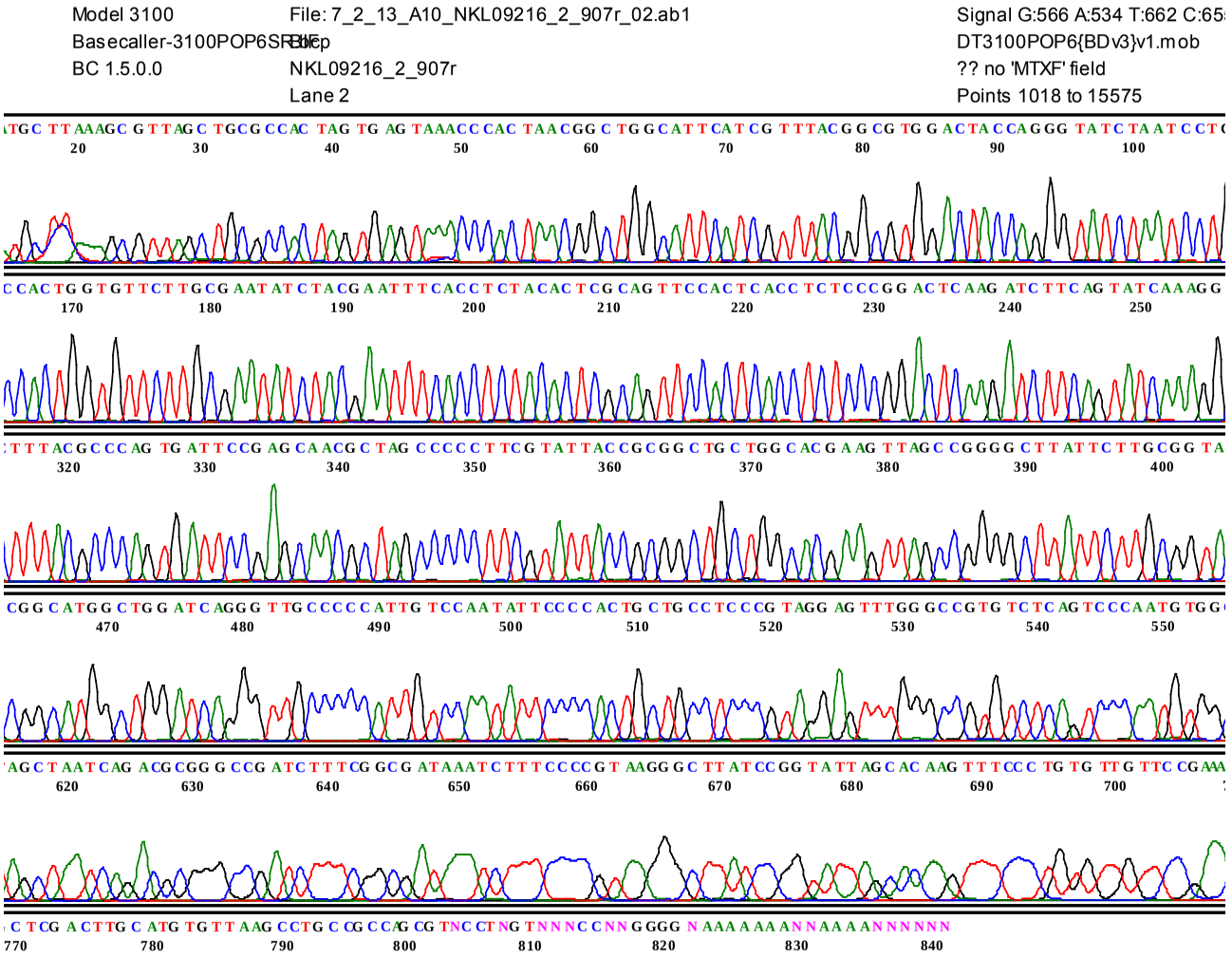
Basecaller-3100POP6SRBC
BC 1.5.0.0
File: 7_2_13_E09_NKL09216_519r_09.ab1
NKL09216_519r
Lane 9
Signal G:198 A:159 T:186 C:18
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1043 to 15575


Model 3100
Basecaller-3100POP6SR
BC 1.5.0.0
File: 7_2_13_G09_NKL09216_787r_13.ab1
NKL09216_787r
Lane 13
Signal G:276 A:258 T:304 C:30
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 956 to 15575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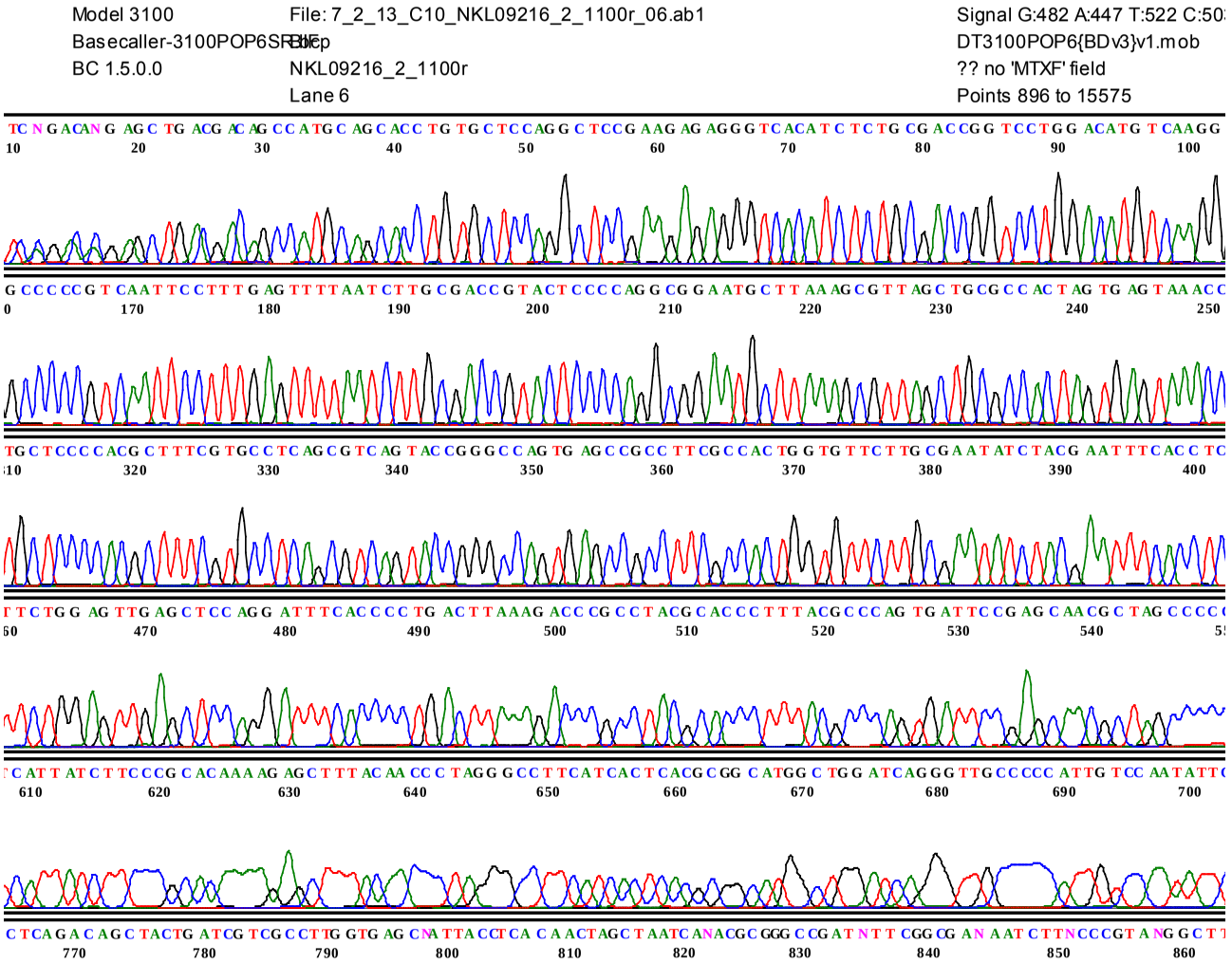
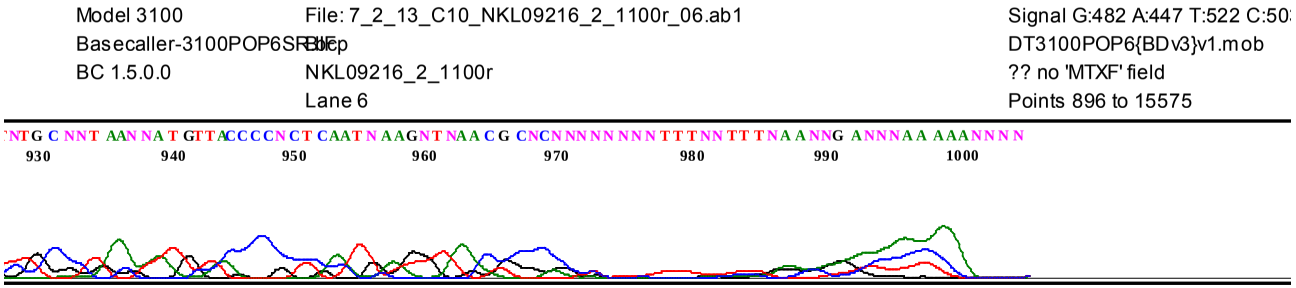
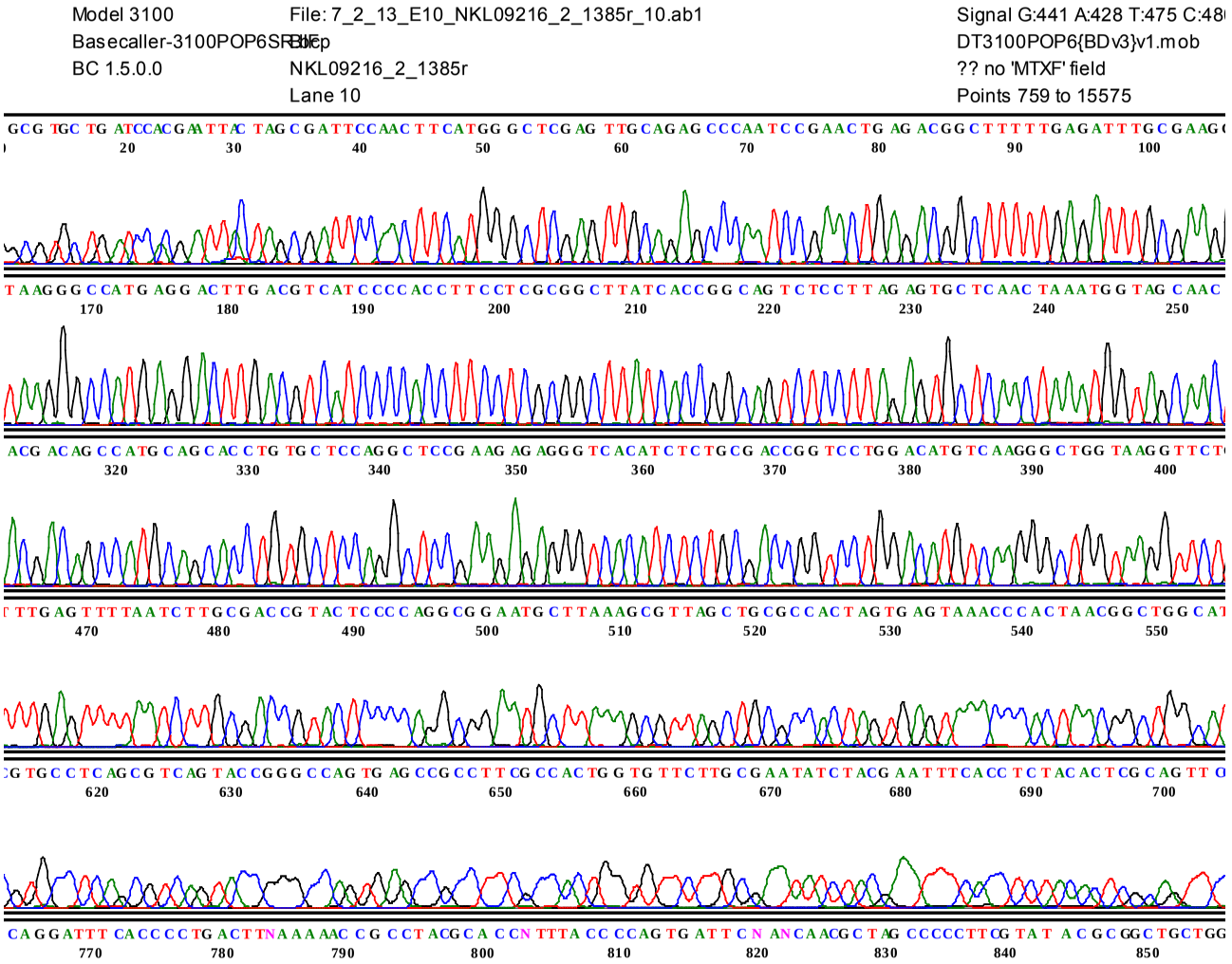
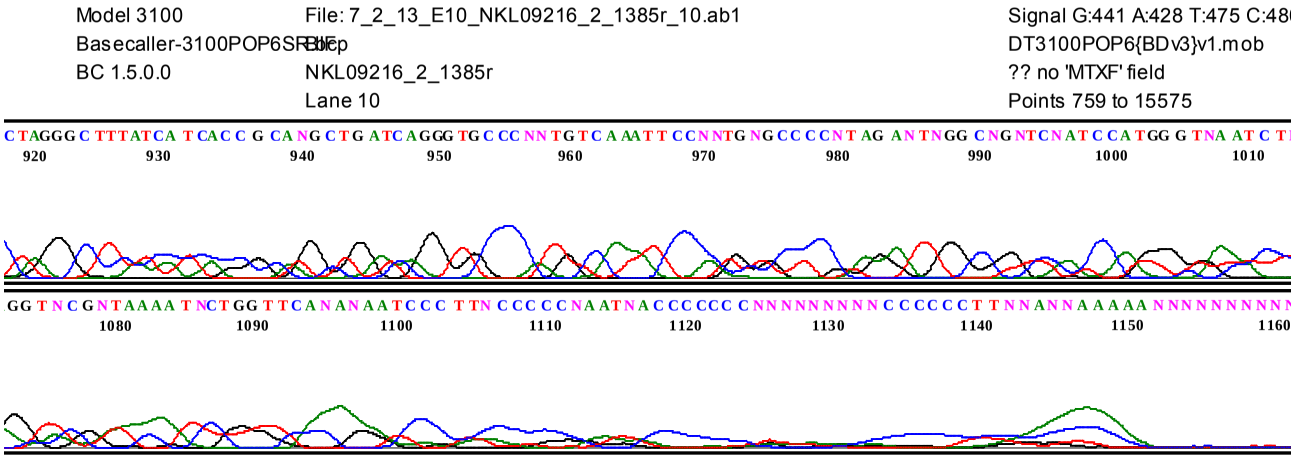
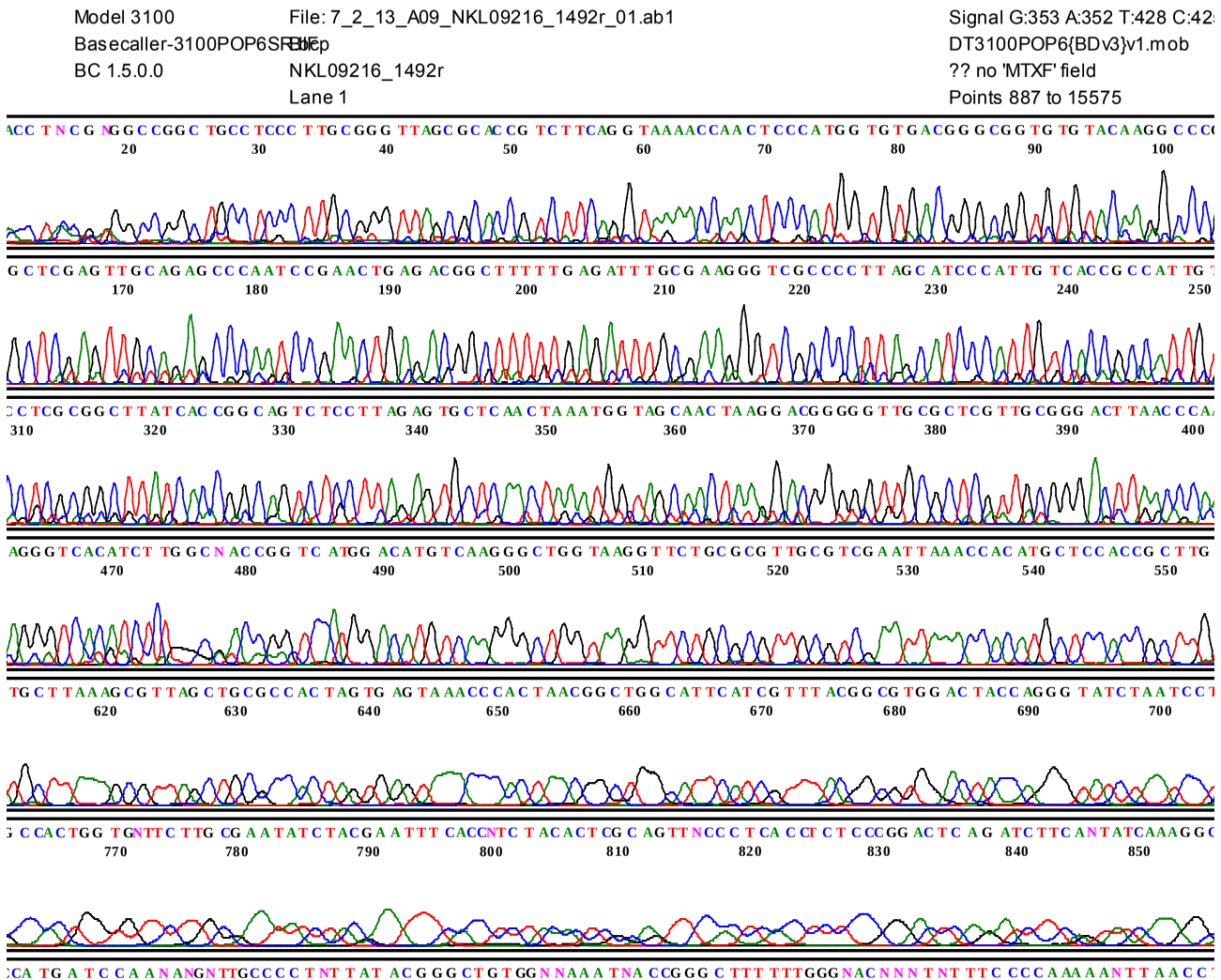
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 7_2_13_A10_NKL09216_2_907r_02.ab1
NKL09216_2_907r
Lane 2
Signal G:566 A:534 T:662 C:65
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1018 to 15575


Model 3100
File: 7_2_13_C10_NKL09216_2_1100r_06.ab1
Signal G:482 A:447 T:522 C:50
Basecaller-3100POP6SRBEp
DT3100POP6{BDv3}v1.mob
BC 1.5.0.0
NKL09216_2_1100r
?? no 'MTXF' field
Lane 6
Points 896 to 15575


Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 7_2_13_C10_NKL09216_2_1100r_06.ab1
NKL09216_2_1100r
Lane 6
Signal G:482 A:447 T:522 C:50
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 896 to 15575
930
940
950
960
970
980
990
1000


Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 7_2_13_E10_NKL09216_2_1385r_10.ab1
NKL09216_2_1385r
Lane 10
Signal G:441 A:428 T:475 C:48
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 759 to 15575


Model 3100
File: 7_2_13_E10_NKL09216_2_1385r_10.ab1
Signal G:441 A:428 T:475 C:48
Basecaller-3100POP6SRBDEp
DT3100POP6{BDv3}v1.mob
BC 1.5.0.0
NKL09216_2_1385r
?? no 'MTXF' field
Lane 10
Points 759 to 15575
920
930
940
950
960
970
980
990
1000
1010
1080
1090
1100
1110
1120
1130
1140
1150
1160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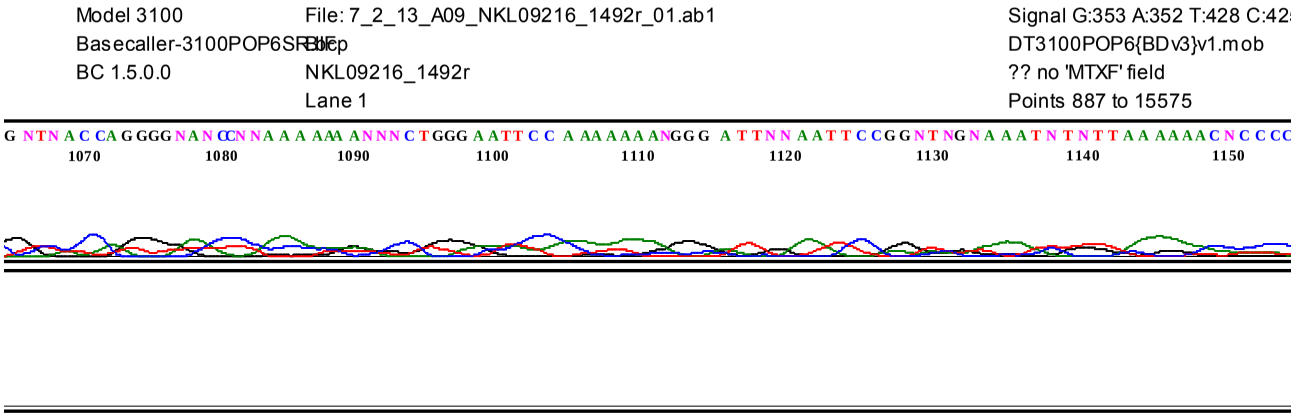
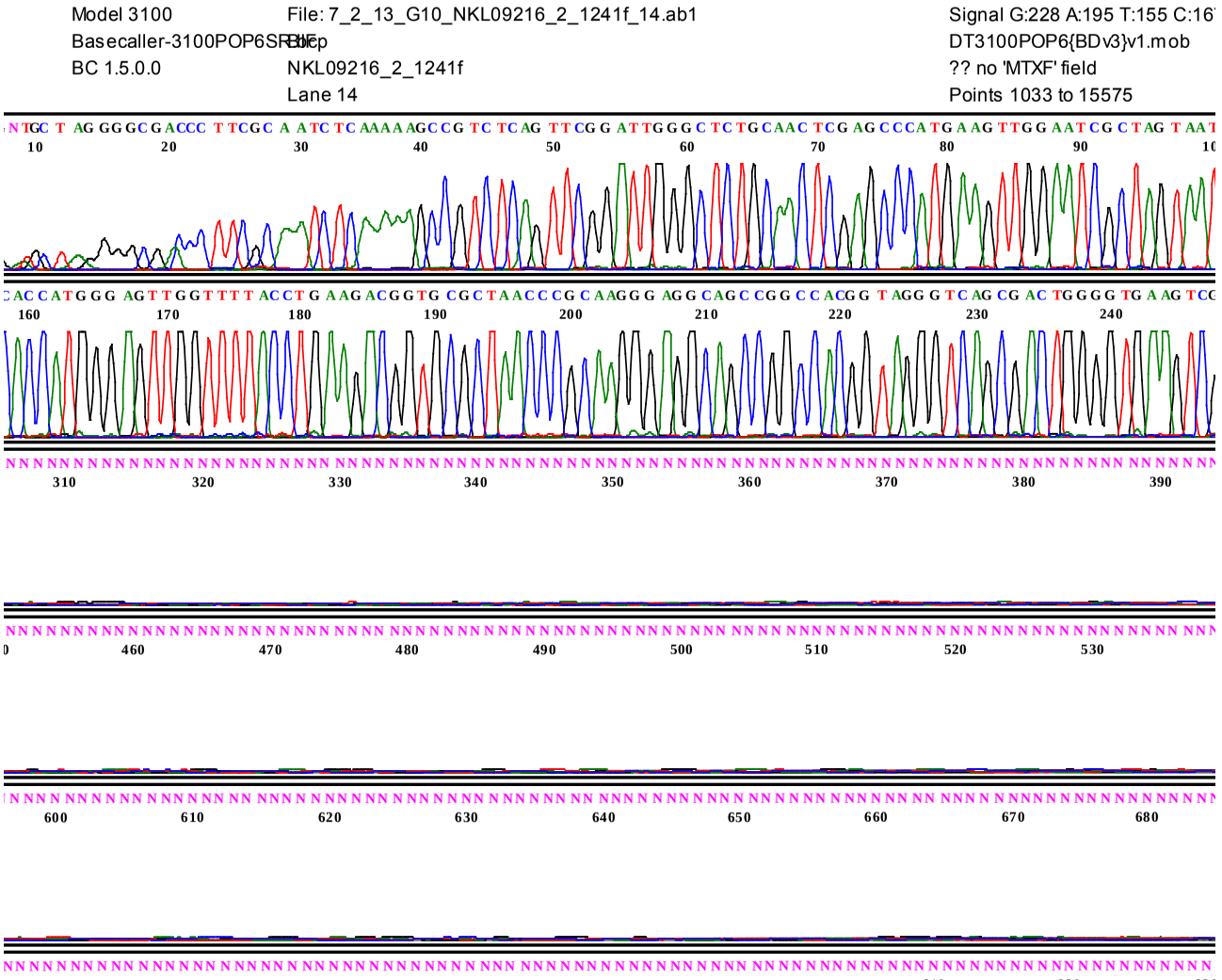
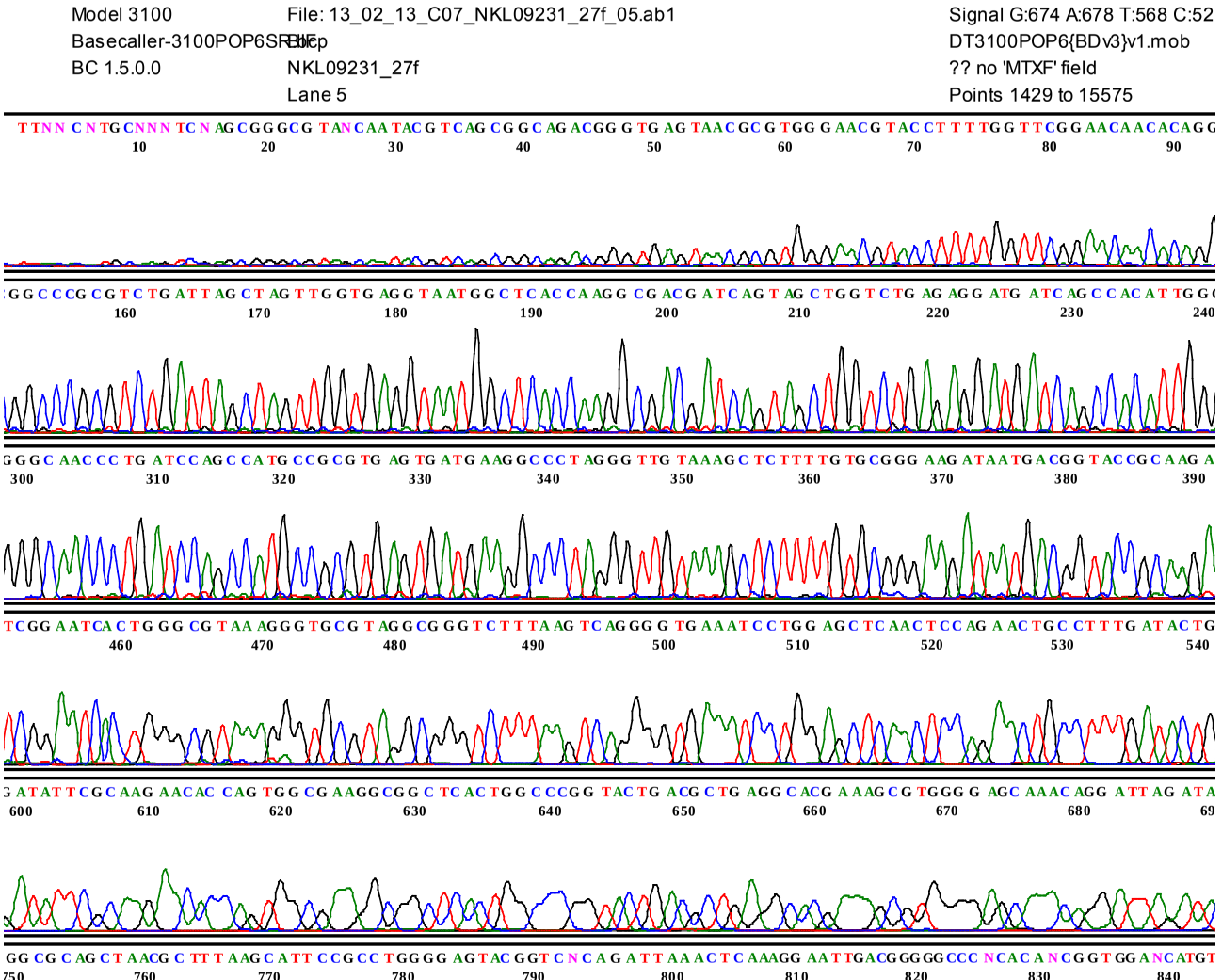
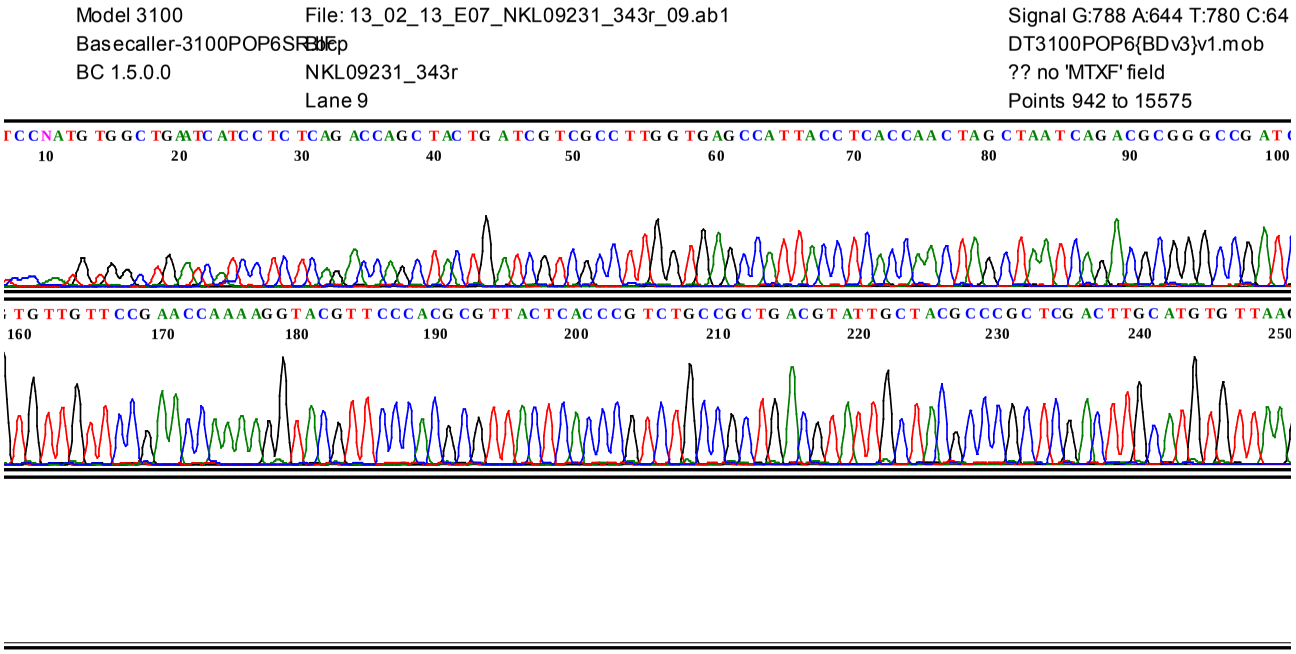
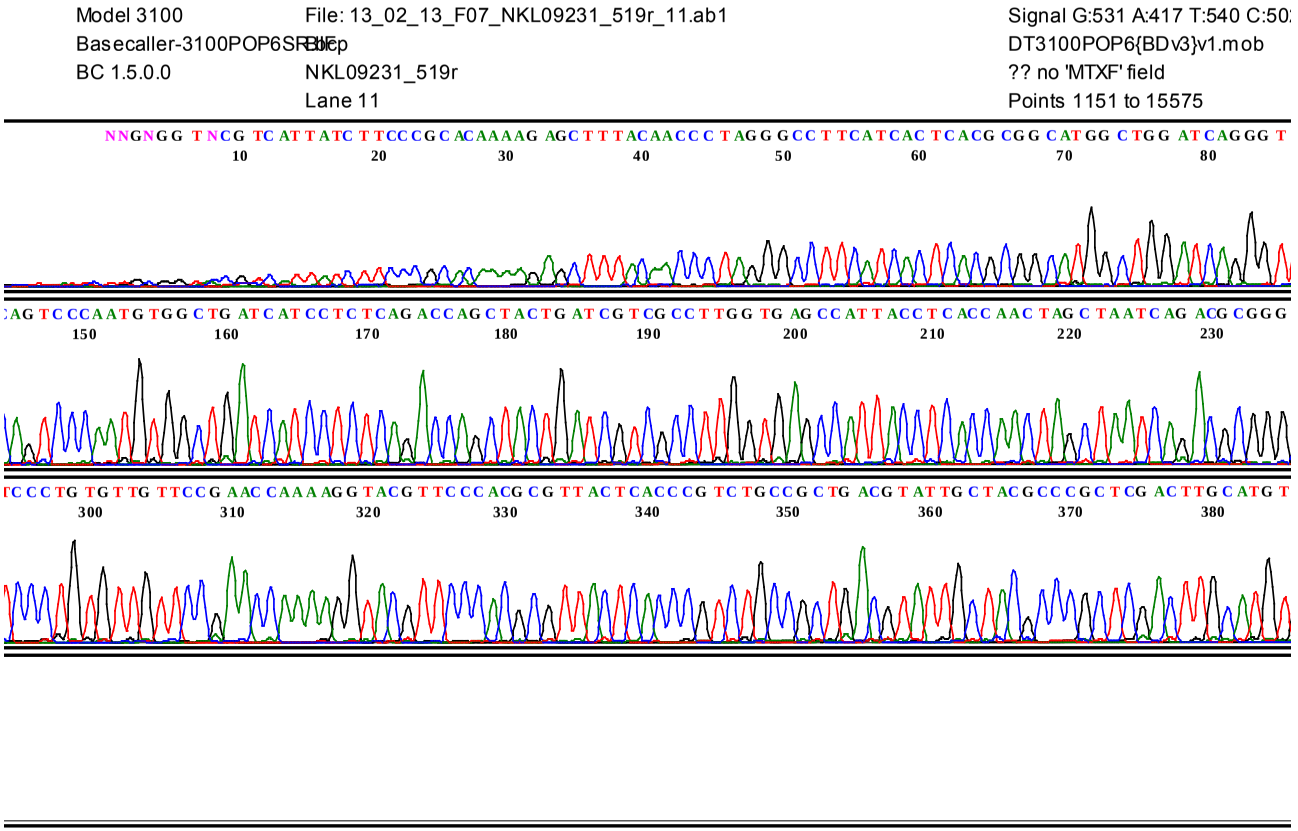
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 7_2_13_A09_NKL09216_1492r_01.ab1
NKL09216_1492r
Lane 1
Signal G:353 A:352 T:428 C:42
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 887 to 15575

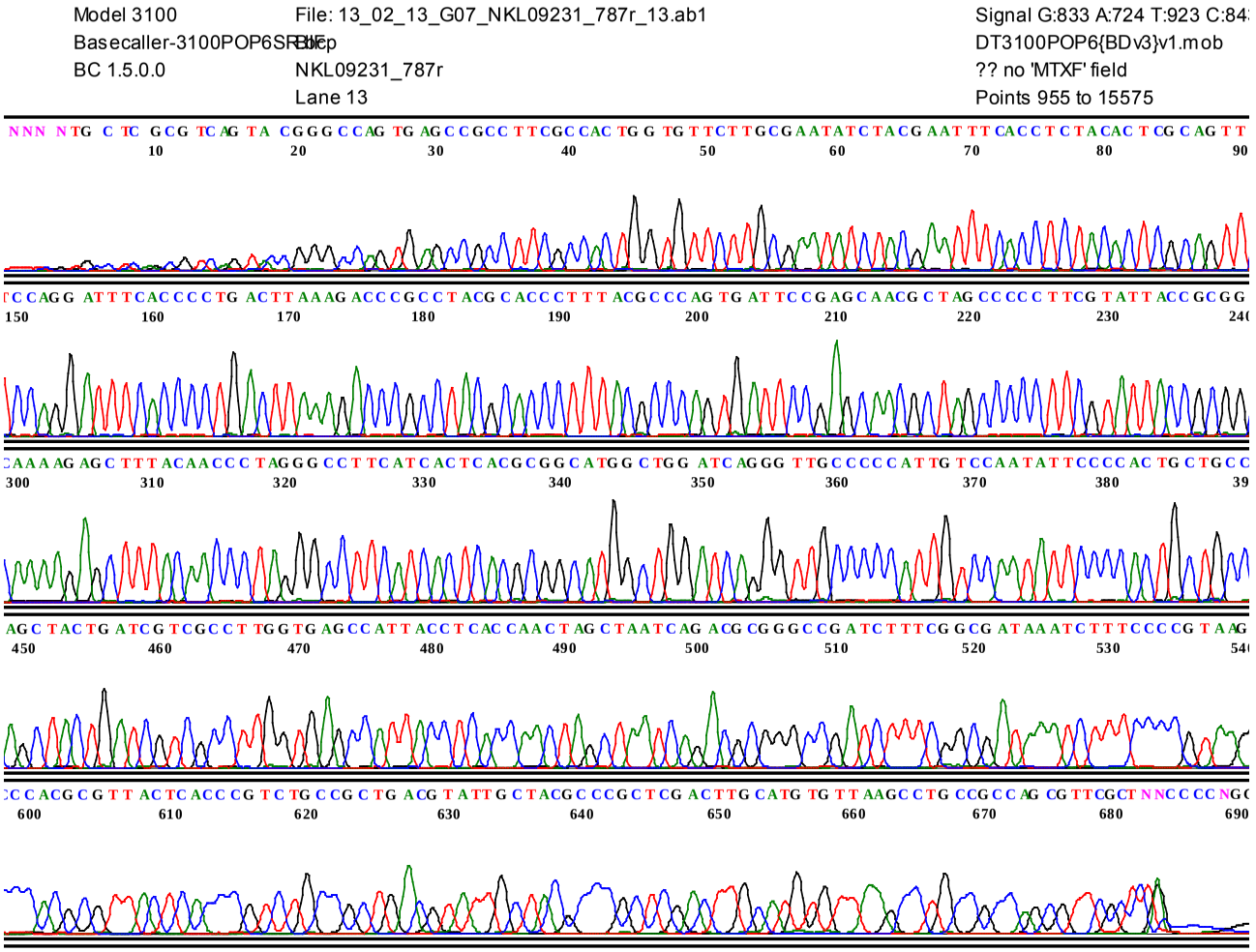

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 7_2_13_A09_NKL09216_1492r_01.ab1
Ep
NKL09216_1492r
Lane 1
Signal G:353 A:352 T:428 C:42
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 887 to 15575
1070
1080
1090
1100
1110
1120
1130
1140
1150

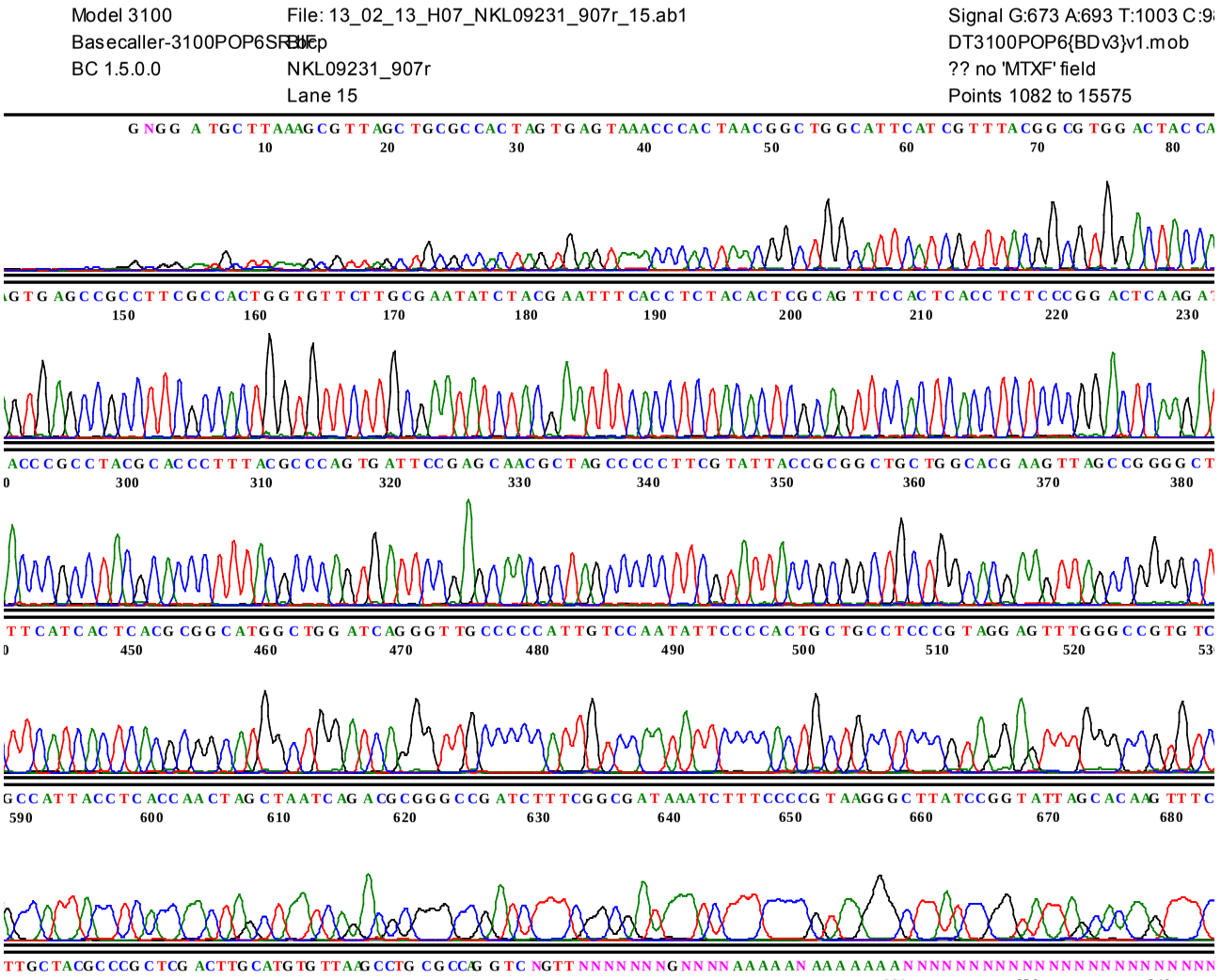

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 7_2_13_G10_NKL09216_2_1241f_14.ab1
NKL09216_2_1241f
Lane 14
Signal G:228 A:195 T:155 C:16
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1033 to 15575

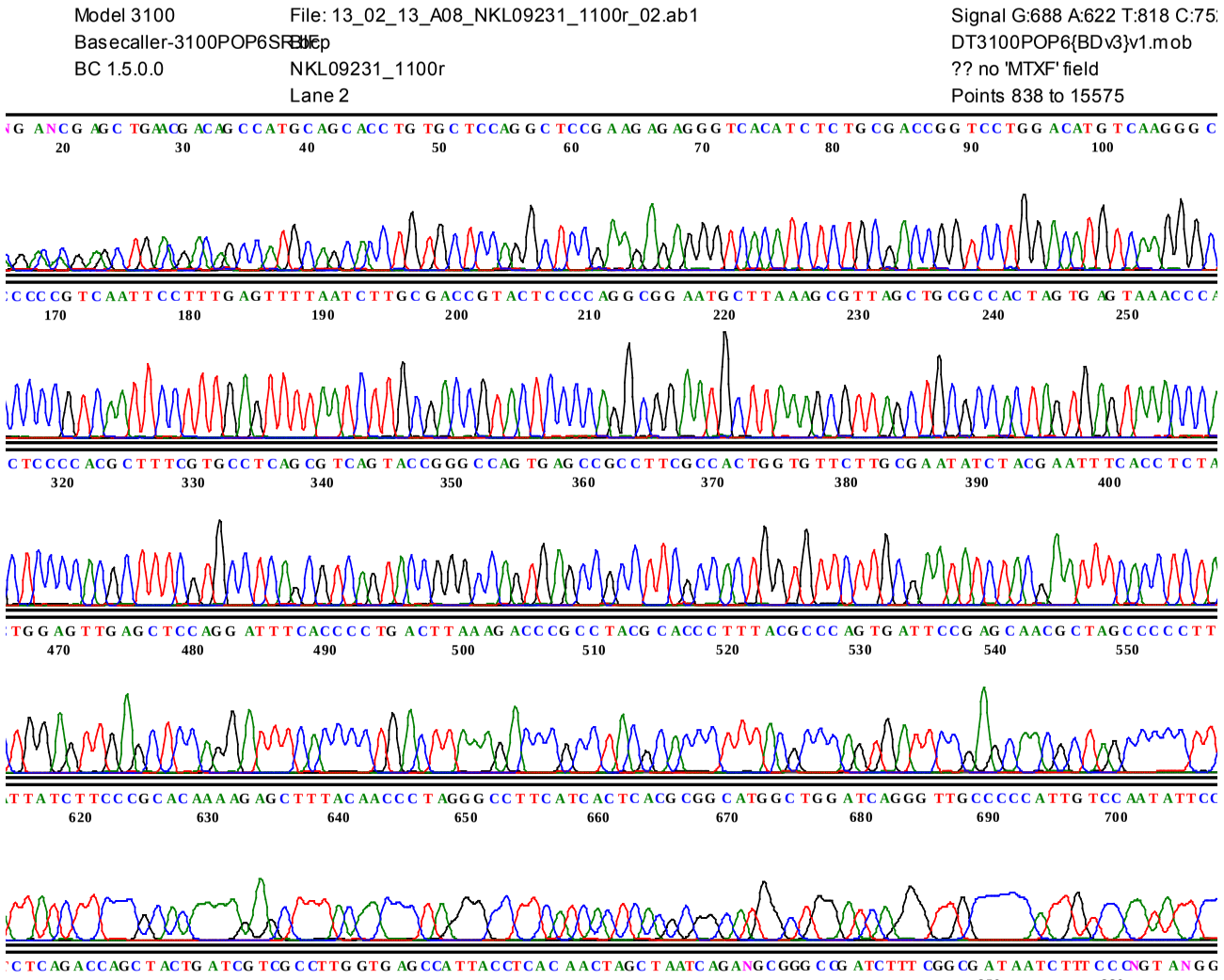

Model 3100
Basecaller-3100POP6SRBDF
BC 1.5.0.0
File: 13_02_13_C07_NKL09231_27f_05.ab1
Ebcp
NKL09231_27f
Lane 5
Signal G:674 A:678 T:568 C:52
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1429 to 15575

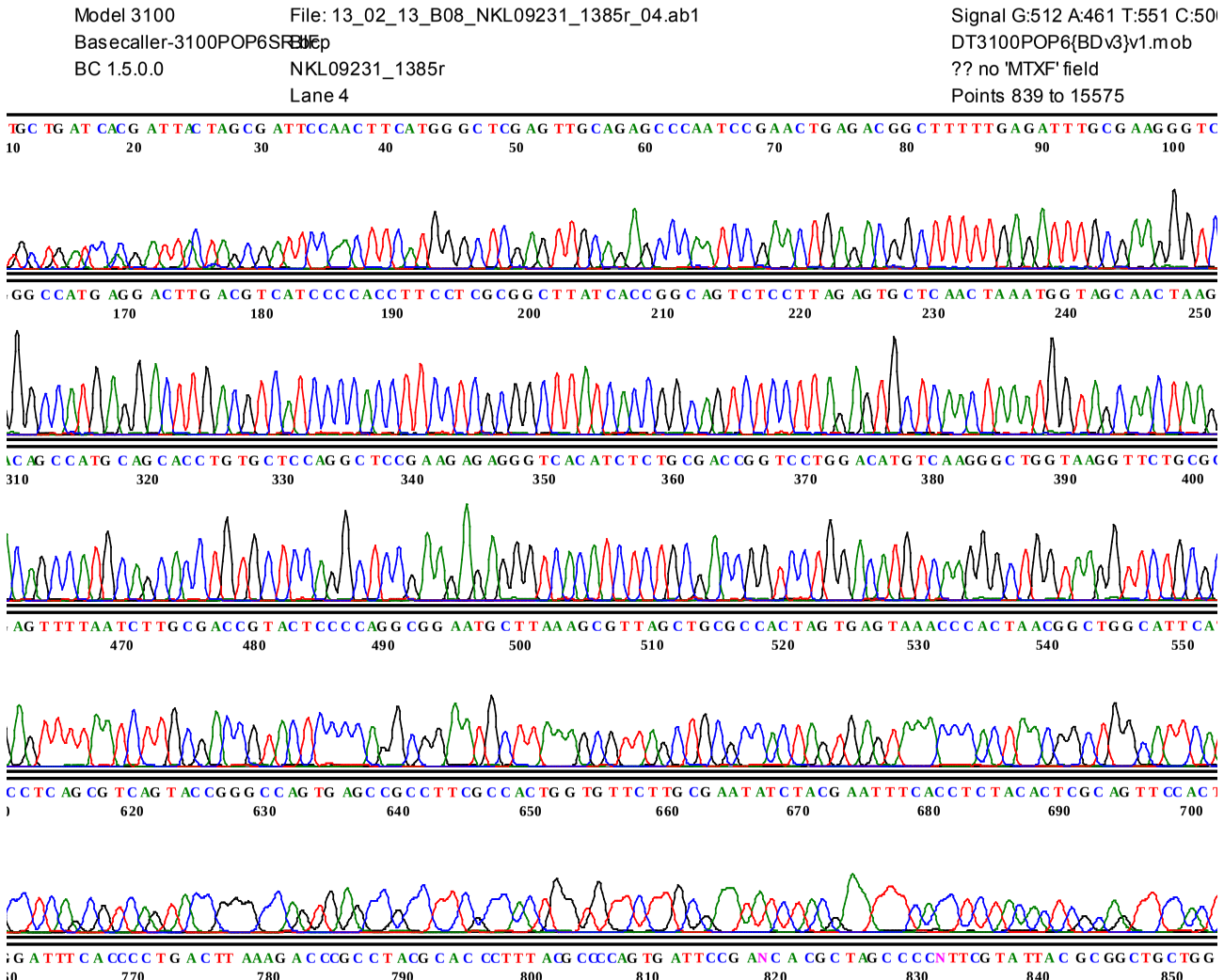

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 13_02_13_E07_NKL09231_343r_09.ab1
NKL09231_343r
Lane 9
Signal G:788 A:644 T:780 C:64
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 942 to 15575

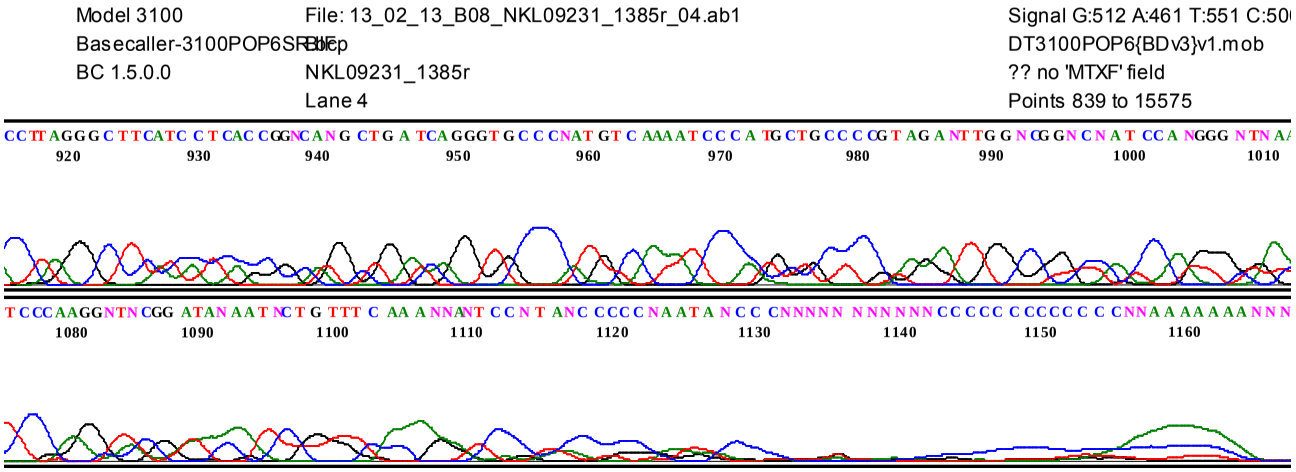

Model 3100
Basecaller-3100POP6SRBC
BC 1.5.0.0
File: 13_02_13_F07_NKL09231_519r_11.ab1
NKL09231_519r
Lane 11
Signal G:531 A:417 T:540 C:50
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1151 to 15575

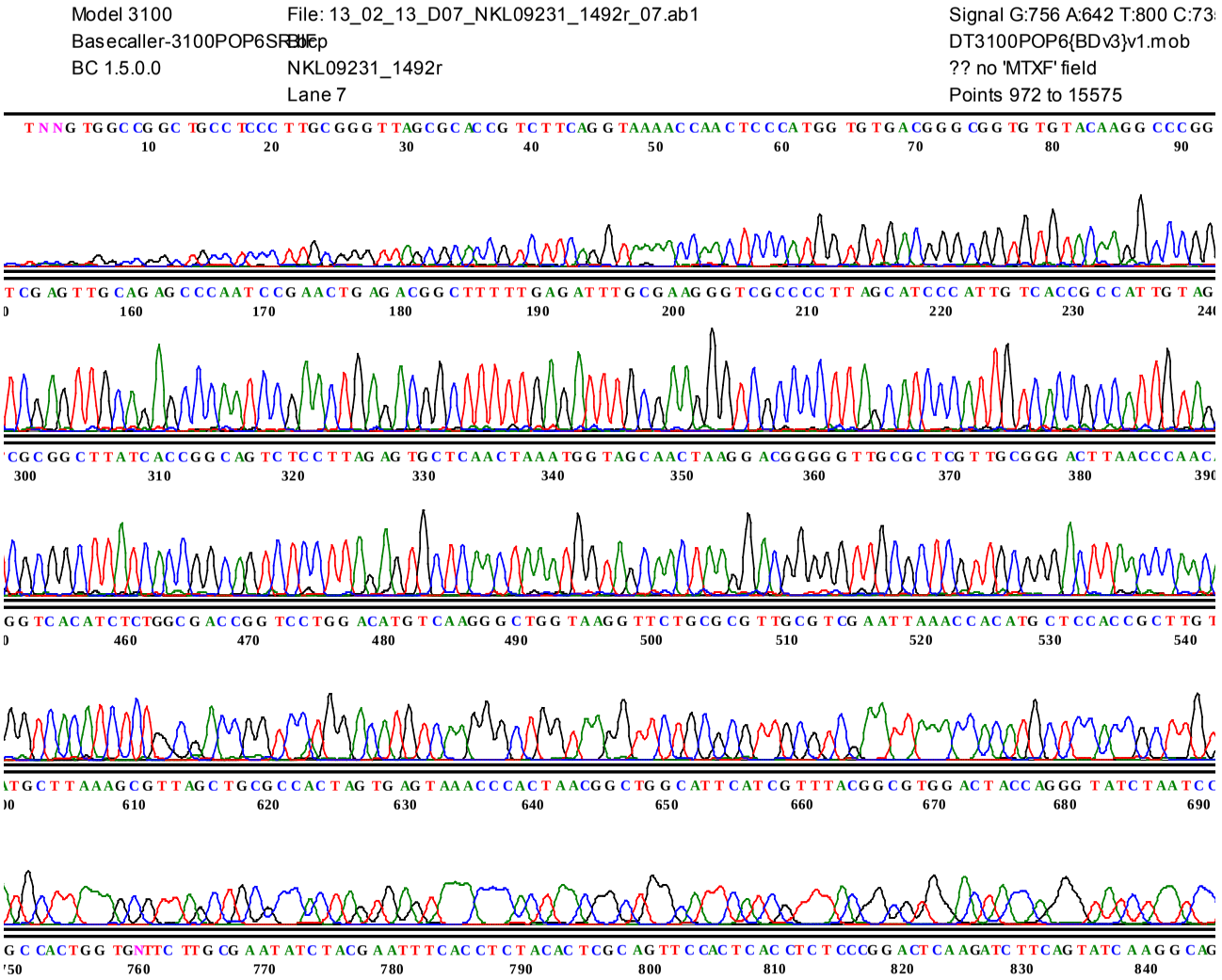

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 13_02_13_G07_NKL09231_787r_13.ab1
NKL09231_787r
Lane 13
Signal G:833 A:724 T:923 C:84
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 955 to 15575

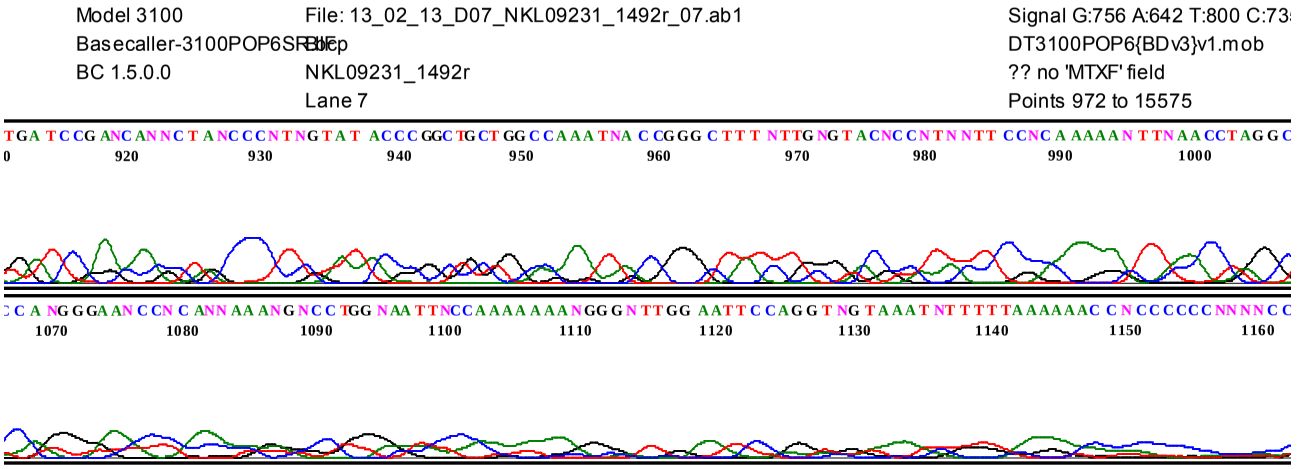

Model 3100
Basecaller-3100POP6SRBC
BC 1.5.0.0
File: 13_02_13_H07_NKL09231_907r_15.ab1
NKL09231_907r
Lane 15
Signal G:673 A:693 T:1003 C:9
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1082 to 15575

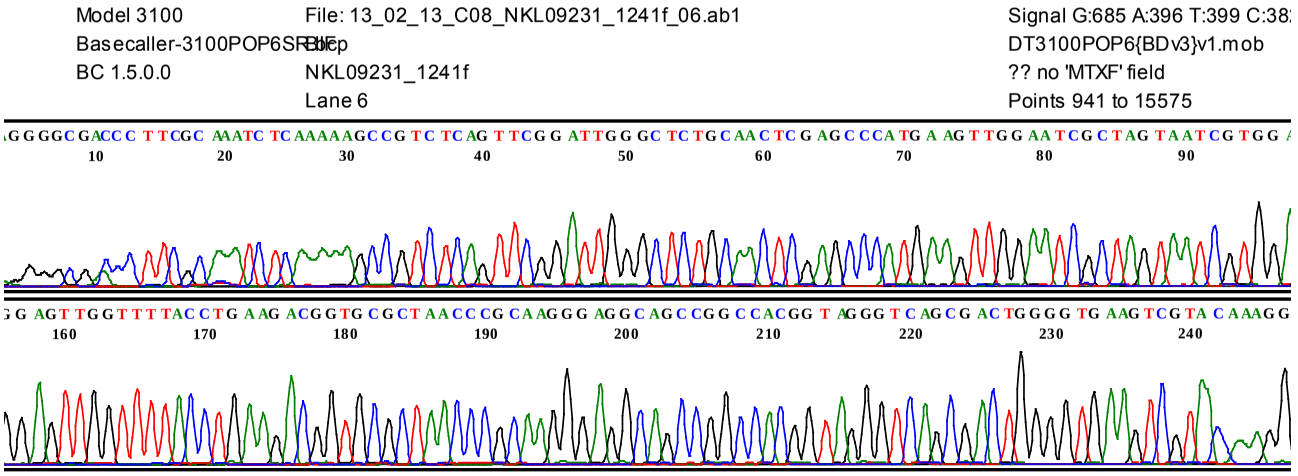

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 13_02_13_A08_NKL09231_1100r_02.ab1
NKL09231_1100r
Lane 2
Signal G:688 A:622 T:818 C:75
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 838 to 15575

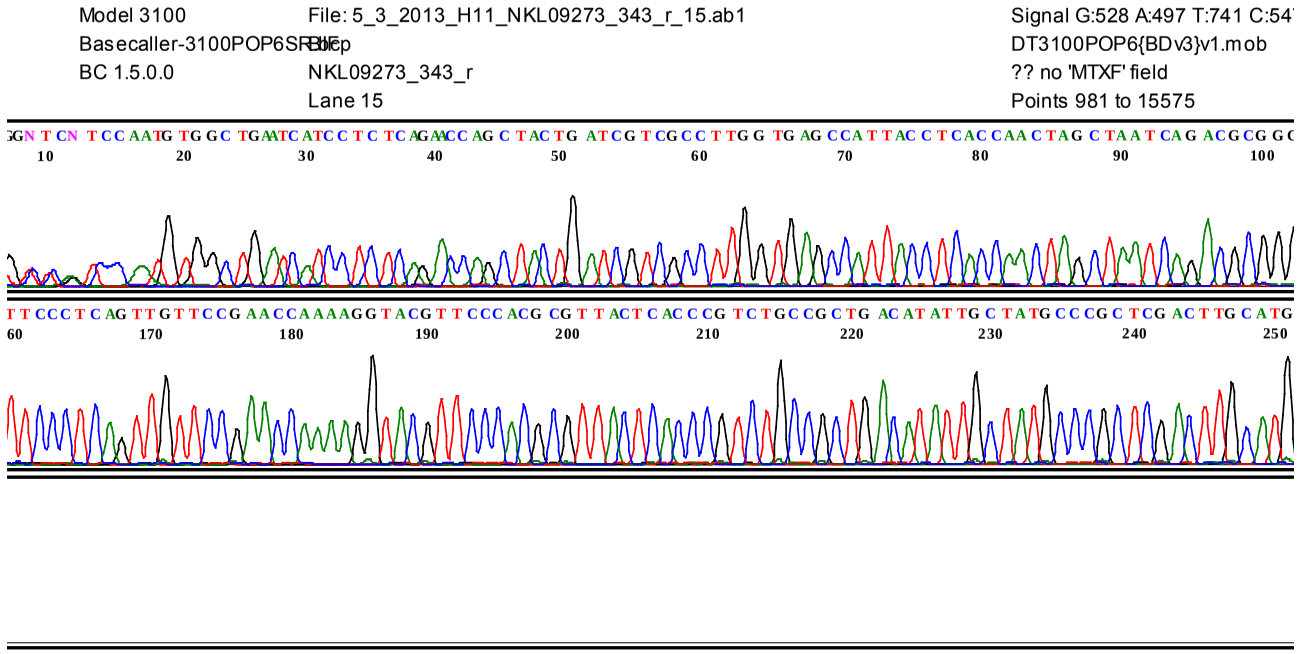

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 13_02_13_B08_NKL09231_1385r_04.ab1
NKL09231_1385r
Lane 4
Signal G:512 A:461 T:551 C:50
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 839 to 15575

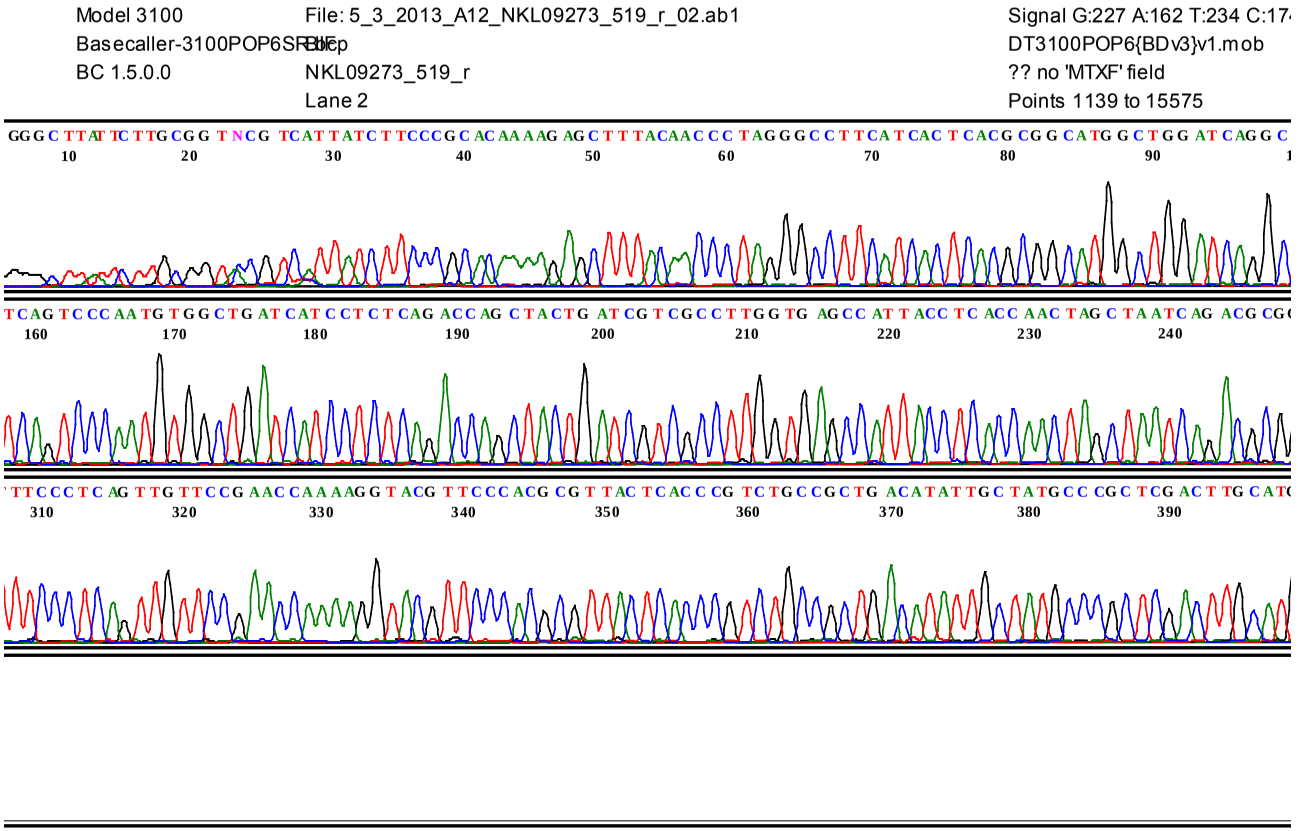

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 13_02_13_B08_NKL09231_1385r_04.ab1
NKL09231_1385r
Lane 4
Signal G:512 A:461 T:551 C:50
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 839 to 15575
920
930
940
950
960
970
980
990
1000
1010
1080
1090
1100
1110
1120
1130
1140
1150
1160

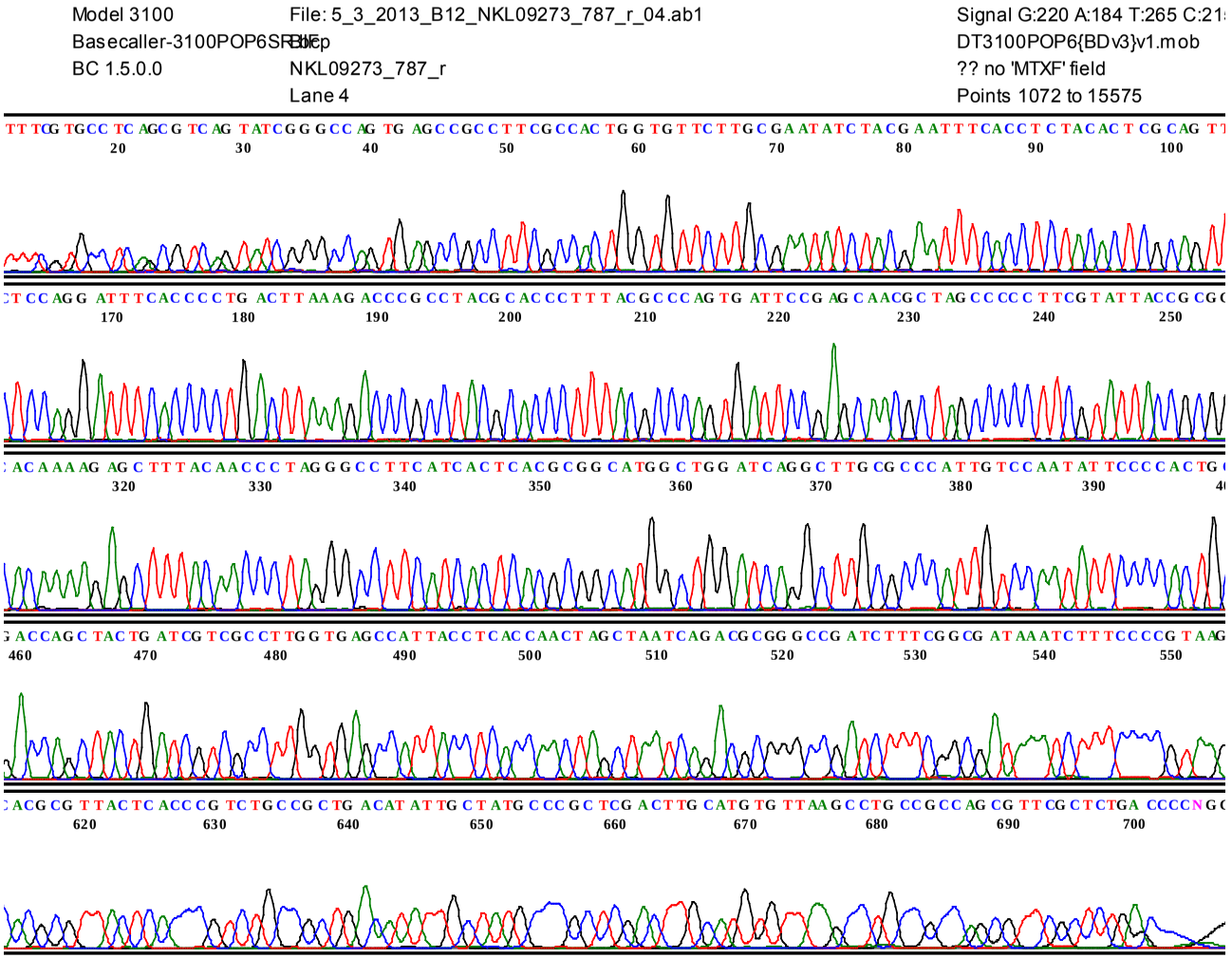

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 13_02_13_D07_NKL09231_1492r_07.ab1
NKL09231_1492r
Lane 7
Signal G:756 A:642 T:800 C:73
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 972 to 15575

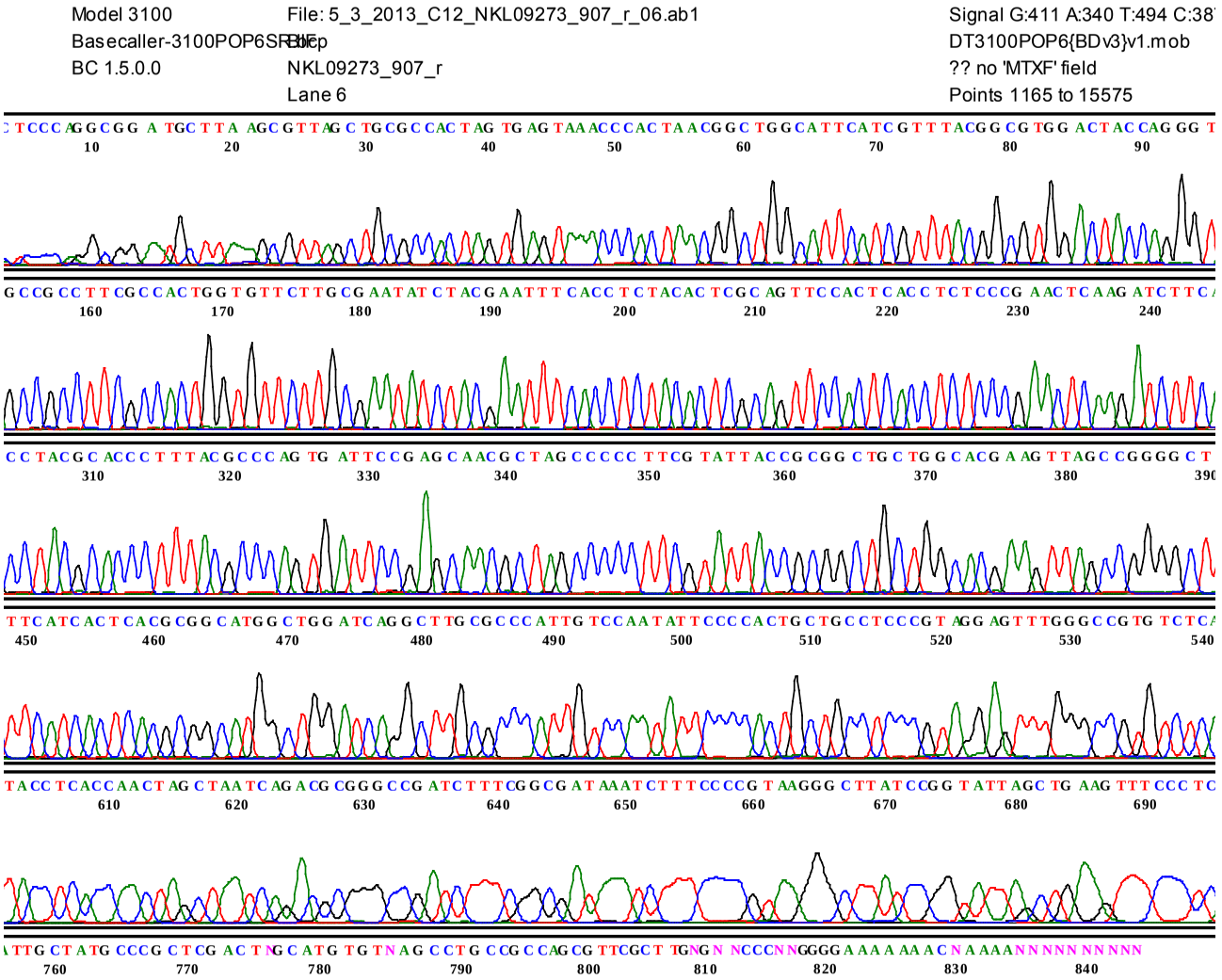

Model 3100
File: 13_02_13_D07_NKL09231_1492r_07.ab1
Signal G:756 A:642 T:800 C:73
Basecaller-3100POP6SRBDtp
DT3100POP6{BDv3}v1.mob
BC 1.5.0.0
NKL09231_1492r
?? no 'MTXF' field
Lane 7
Points 972 to 15575
0
920
930
940
950
960
970
980
990
1000
1070
1080
1090
1100
1110
1120
1130
1140
1150
1160

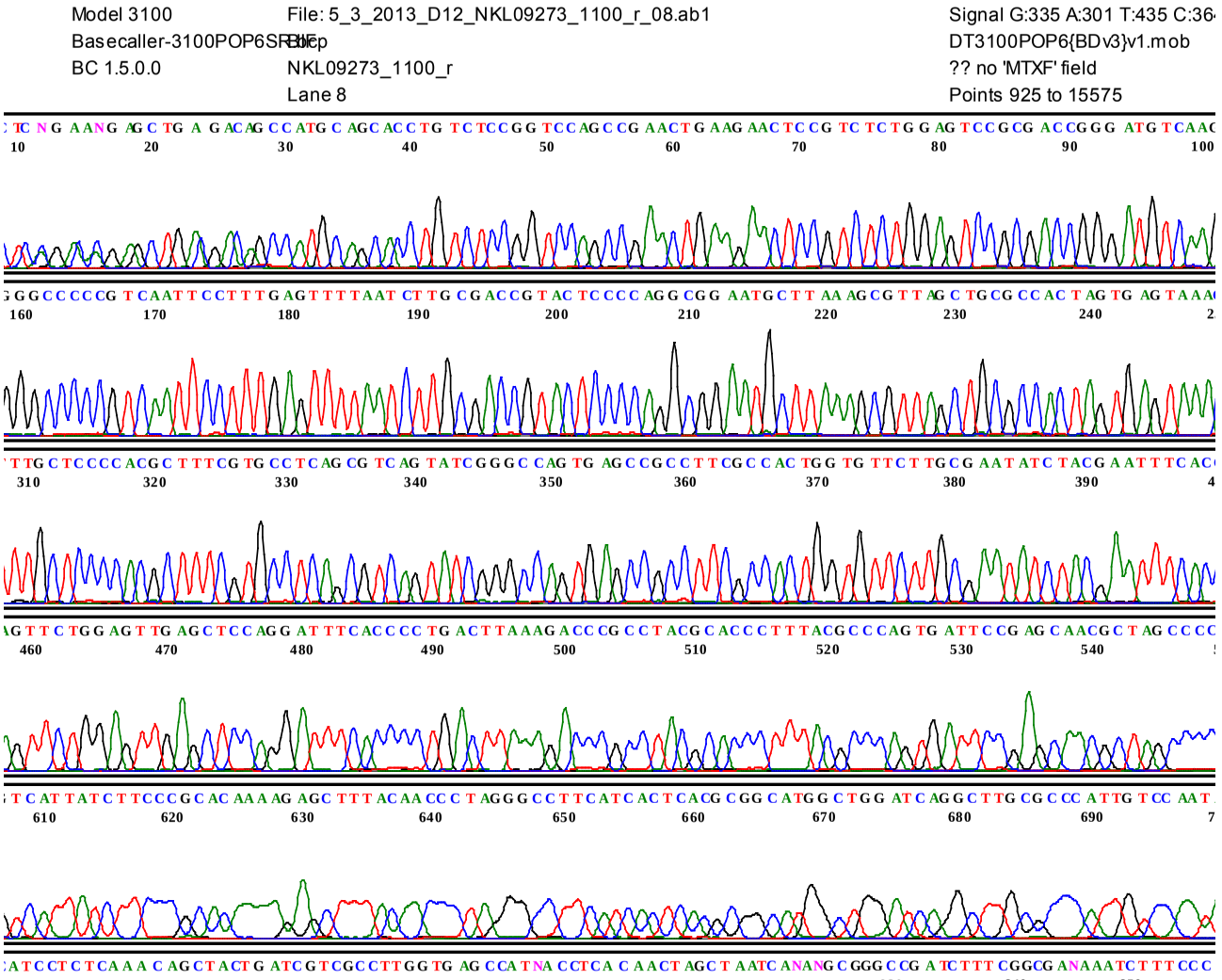

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 13_02_13_C08_NKL09231_1241f_06.ab1
NKL09231_1241f
Lane 6
Signal G:685 A:396 T:399 C:38
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 941 to 15575

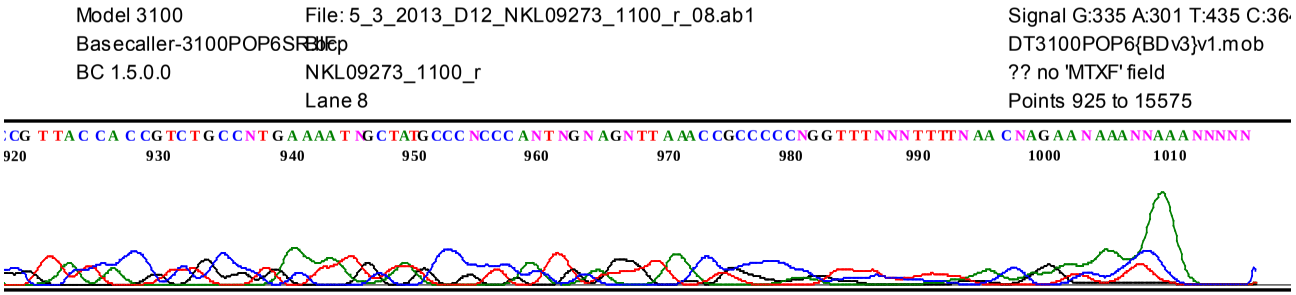

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 5_3_2013_H11_NKL09273_343_r_15.ab1
NKL09273_343_r
Lane 15
Signal G:528 A:497 T:741 C:54
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 981 to 15575
GN T CN T CC AATG TG GC TGAATC ATCC T C TC AGAACC AG C T AC TG ATCG T CG CC T TG G TG AG CCA T T AC C T C AC CAA C T AG C T A A T CA G ACG CG G C
10 20 30 40 50 60 70 80 90 100
T T CCC T C AG TT G T T C CG AA CC AAAAGG T A CG T T CCC ACG CG T T A CT C A CC C CG T C T GC C G C T G AC AT AT T G C T A T G C C C G C T C G AC T T G C A T G
60 170 180 190 200 210 220 230 240 250

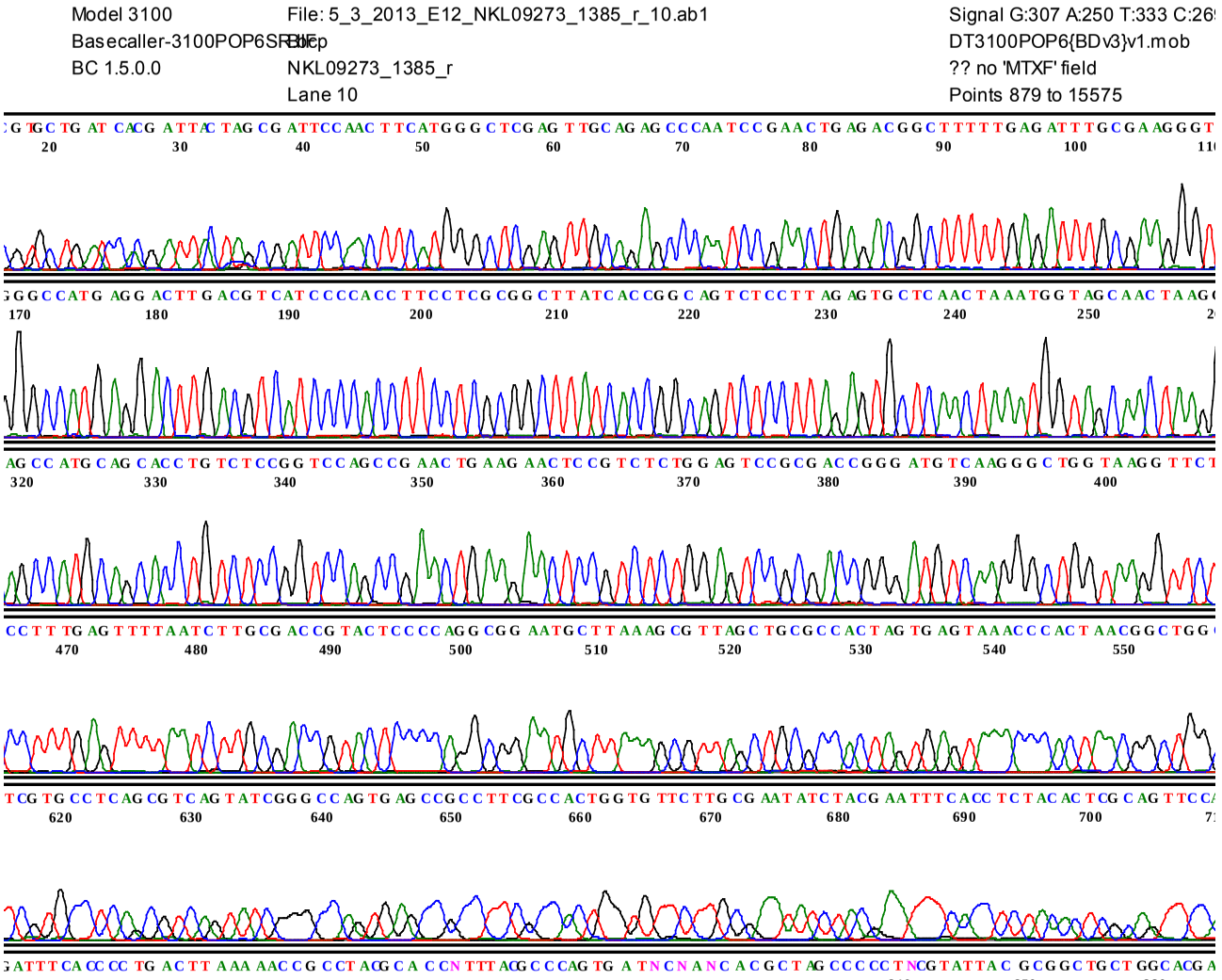

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 5_3_2013_A12_NKL09273_519_r_02.ab1
NKL09273_519_r
Lane 2
Signal G:227 A:162 T:234 C:17
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1139 to 15575

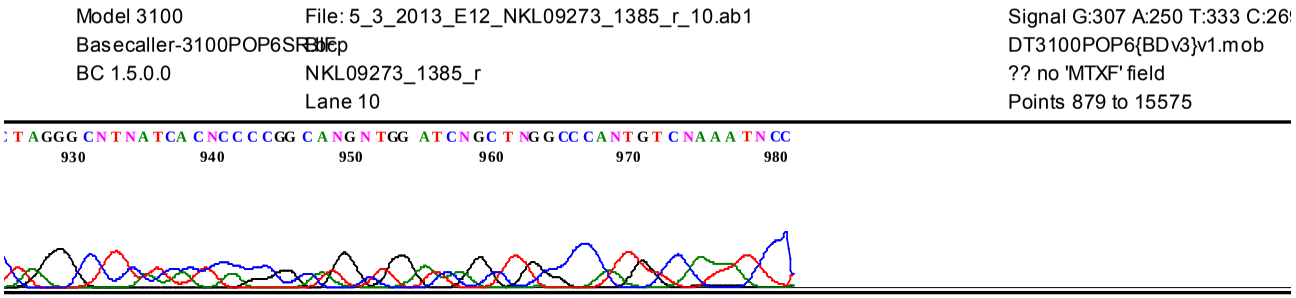

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 5_3_2013_B12_NKL09273_787_r_04.ab1
NKL09273_787_r
Lane 4
Signal G:220 A:184 T:265 C:21
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1072 to 15575

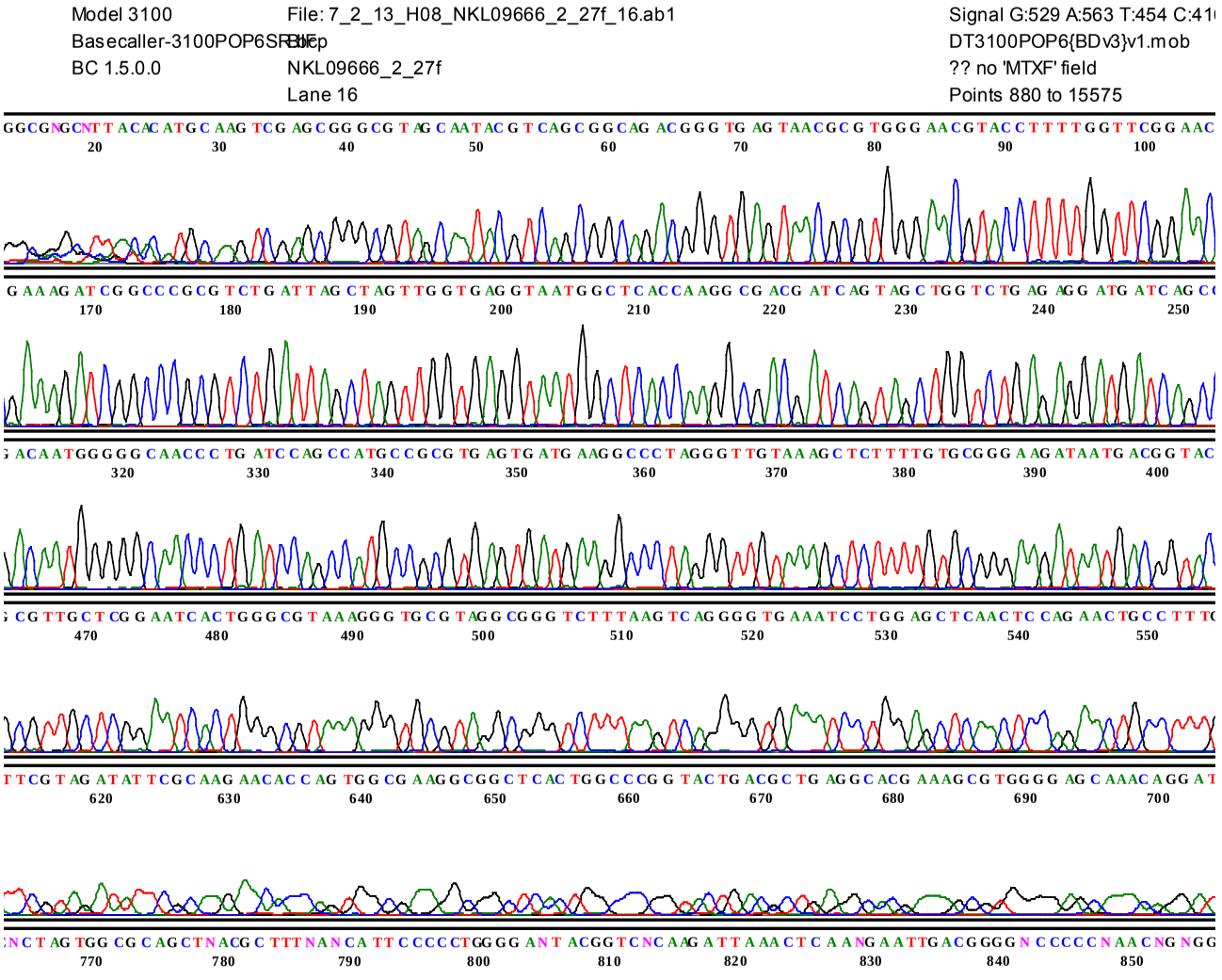

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 5_3_2013_C12_NKL09273_907_r_06.ab1
NKL09273_907_r
Lane 6
Signal G:411 A:340 T:494 C:38
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1165 to 15575

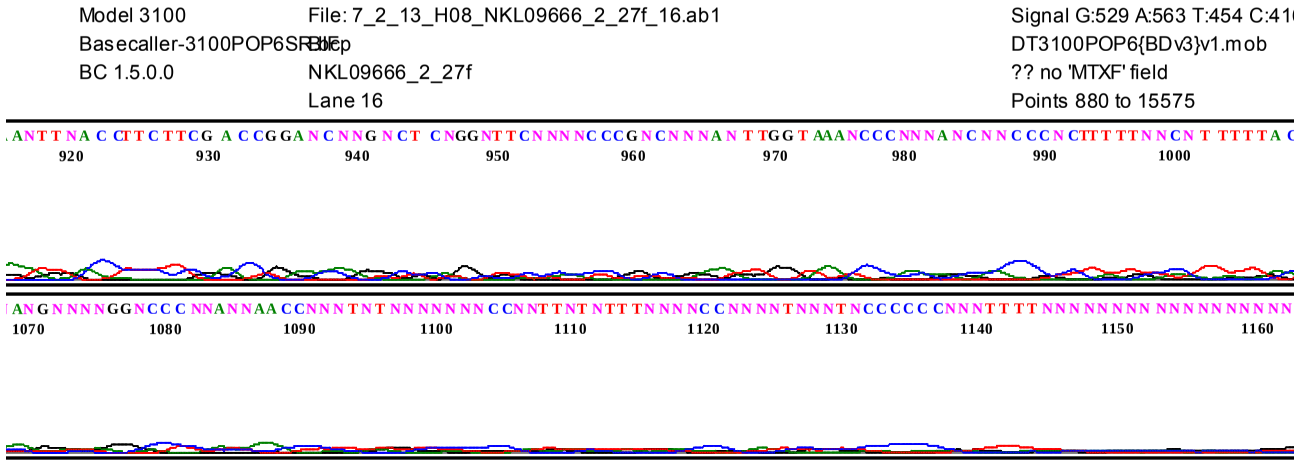

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 5_3_2013_D12_NKL09273_1100_r_08.ab1
NKL09273_1100_r
Lane 8
Signal G:335 A:301 T:435 C:36
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 925 to 15575

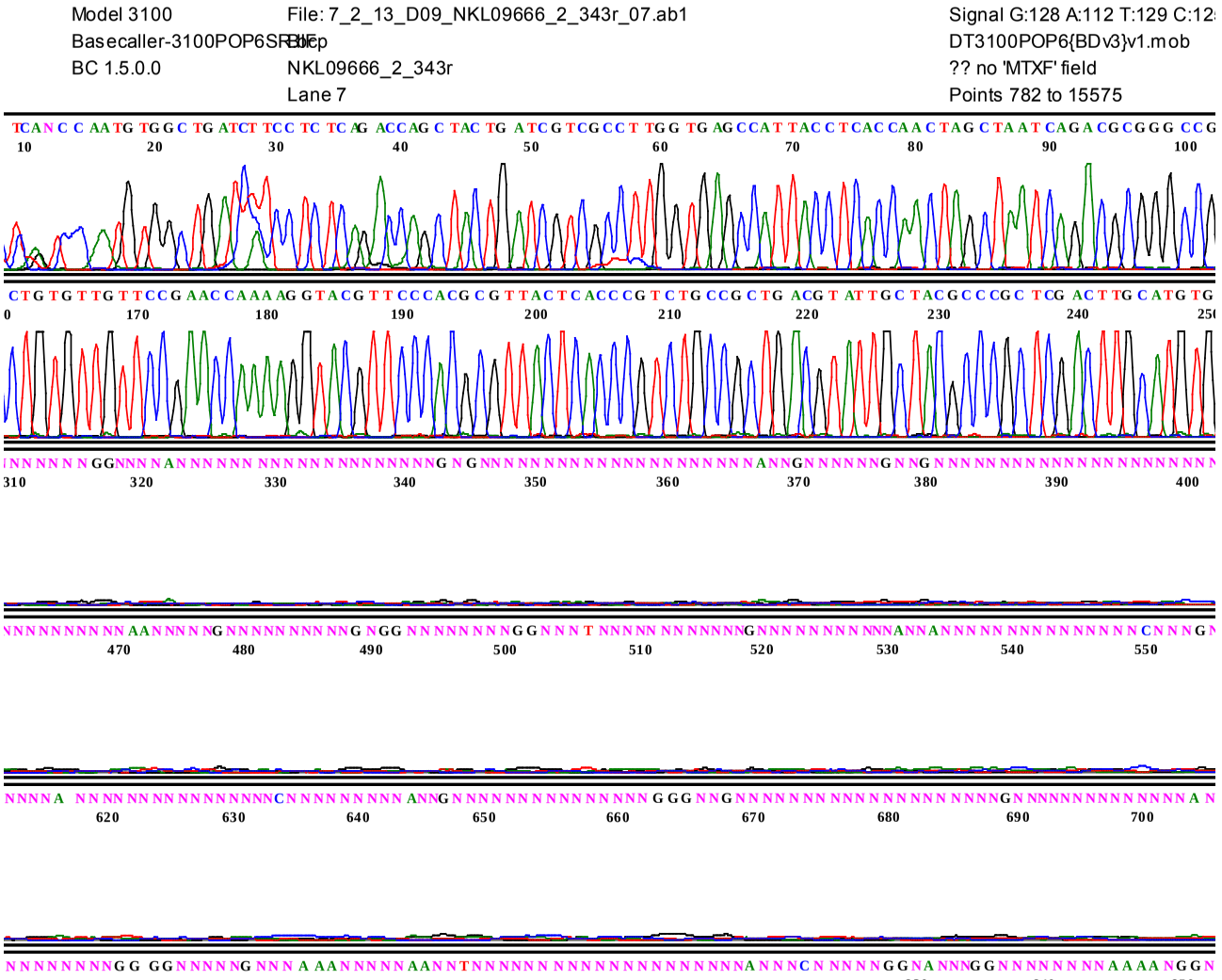

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 5_3_2013_D12_NKL09273_1100_r_08.ab1
Ep
NKL09273_1100_r
Lane 8
Signal G:335 A:301 T:435 C:36
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 925 to 15575
CG T TA C CA C CG TC T G C C NT G A AAA T NG C TATG CCC NCCC A NT NG N AG N TT A AA C C GC C C C C NG G T T T NNN T T TT N AA C NA G A A N AAA NNA A A NNNNN
920
930
940
950
960
970
980
990
1000
1010

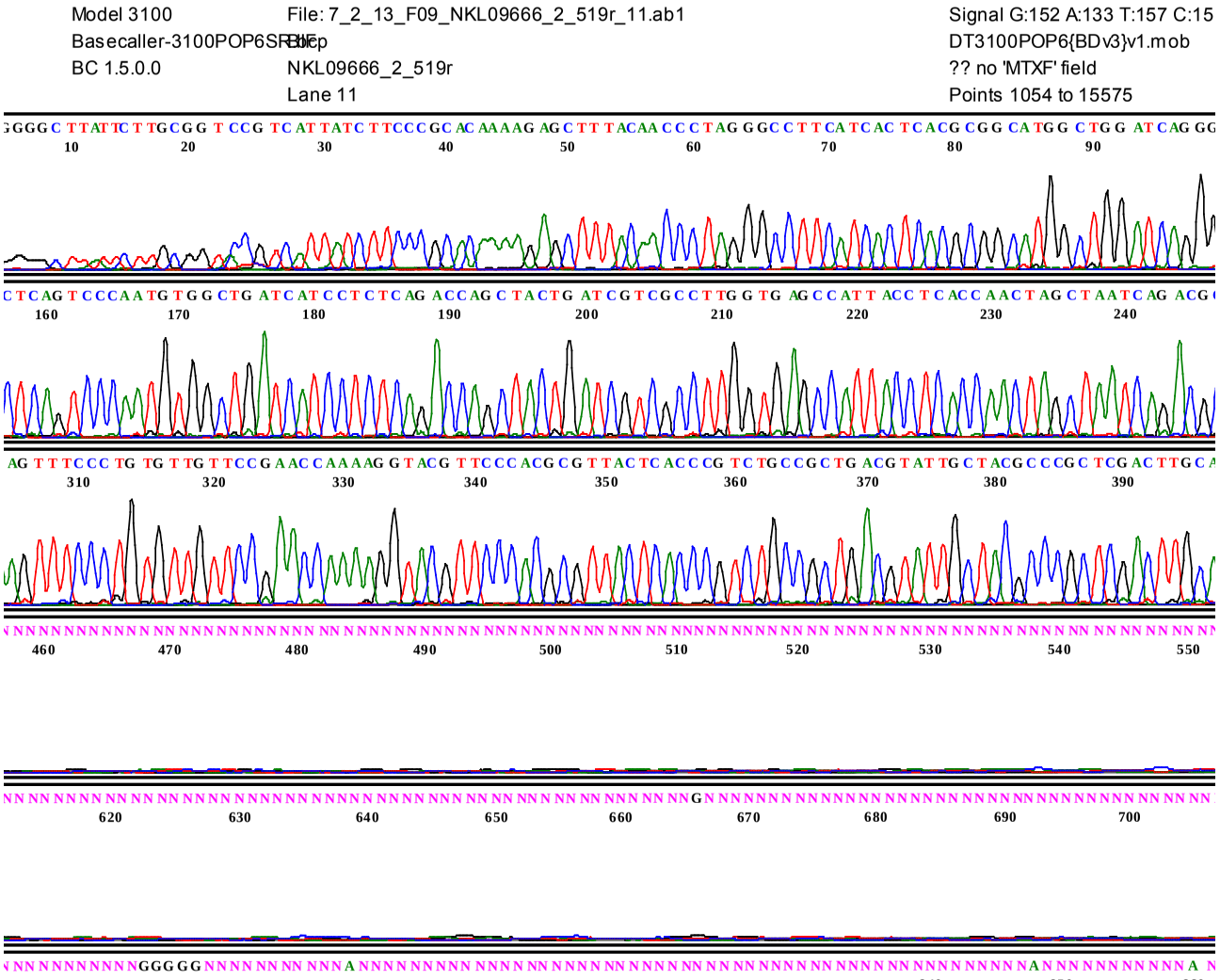

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 5_3_2013_E12_NKL09273_1385_r_10.ab1
Ep
NKL09273_1385_r
Lane 10
Signal G:307 A:250 T:333 C:26
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 879 to 15575

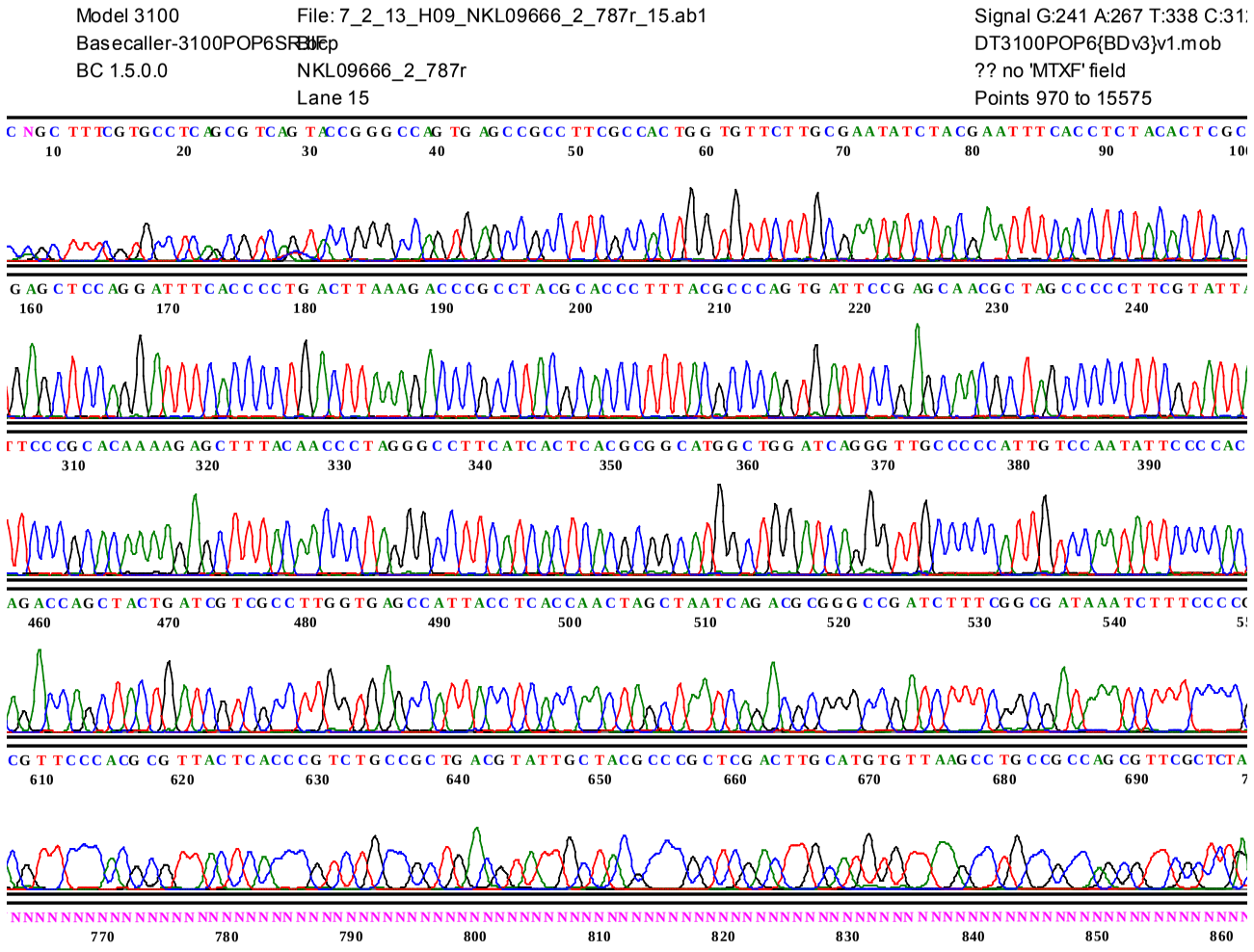

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 5_3_2013_E12_NKL09273_1385_r_10.ab1
Ep
NKL09273_1385_r
Lane 10
Signal G:307 A:250 T:333 C:26
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 879 to 15575
T AGGG CNTNA TCA CNCC C CGG C A NG N TGG ATC NGC T NG G CC CA NT G T C NA A A TN CC
930
940
950
960
970
980

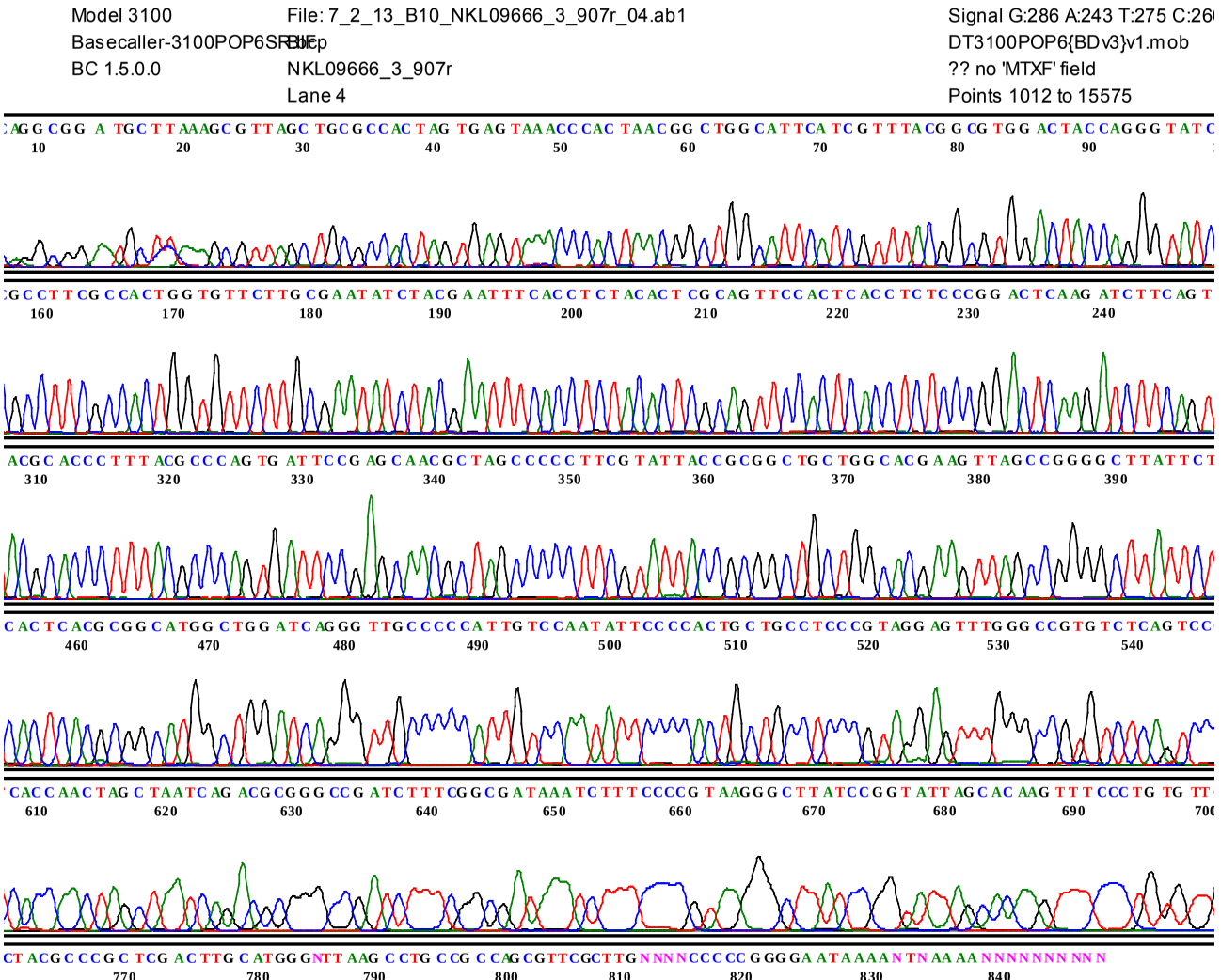

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 7_2_13_H08_NKL09666_2_27f_16.ab1
NKL09666_2_27f
Lane 16
Signal G:529 A:563 T:454 C:41
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 880 to 15575

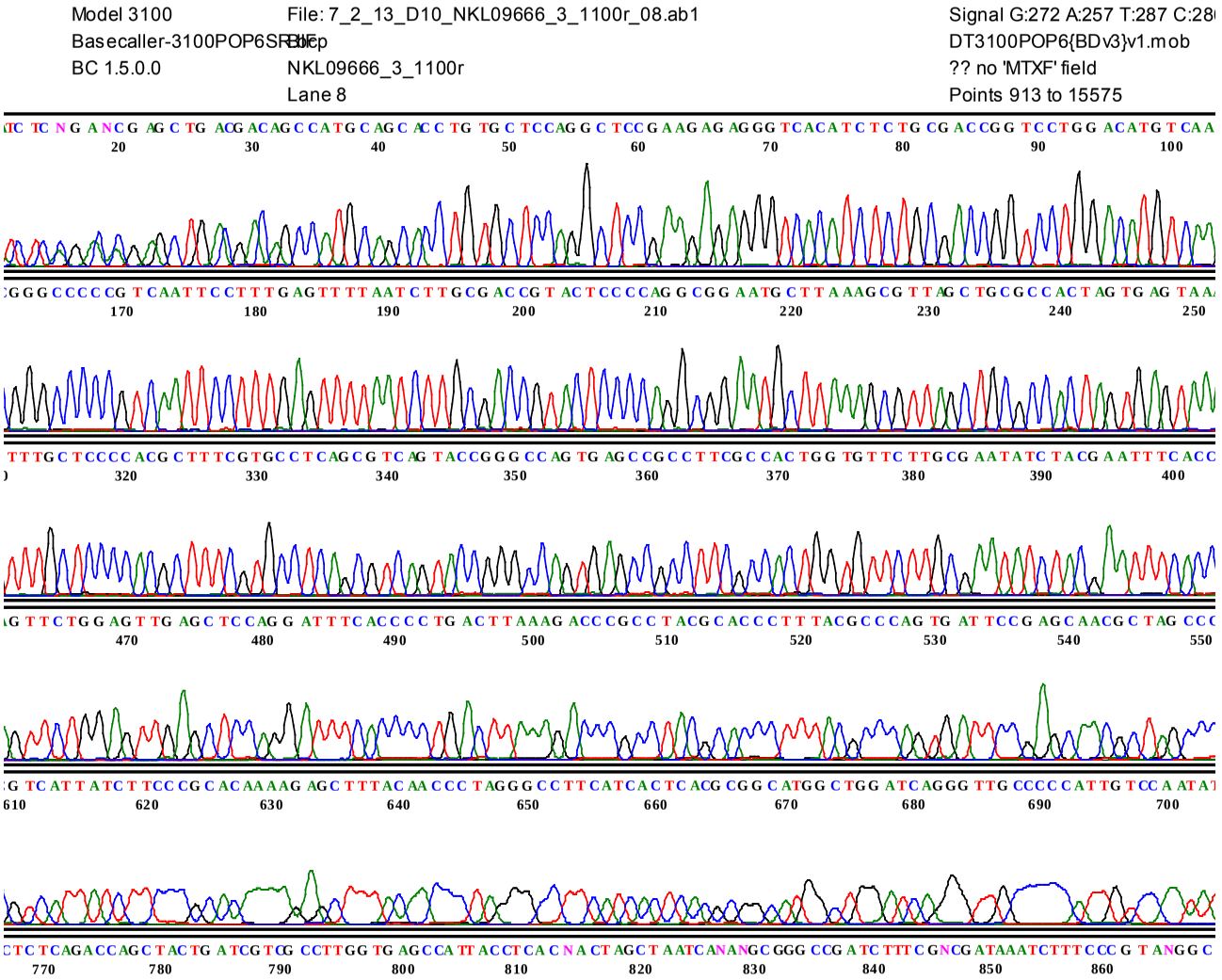

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 7_2_13_H08_NKL09666_2_27f_16.ab1
Ecp
NKL09666_2_27f
Lane 16
Signal G:529 A:563 T:454 C:41
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 880 to 15575
920
930
940
950
960
970
980
990
1000
1070
1080
1090
1100
1110
1120
1130
1140
1150
1160

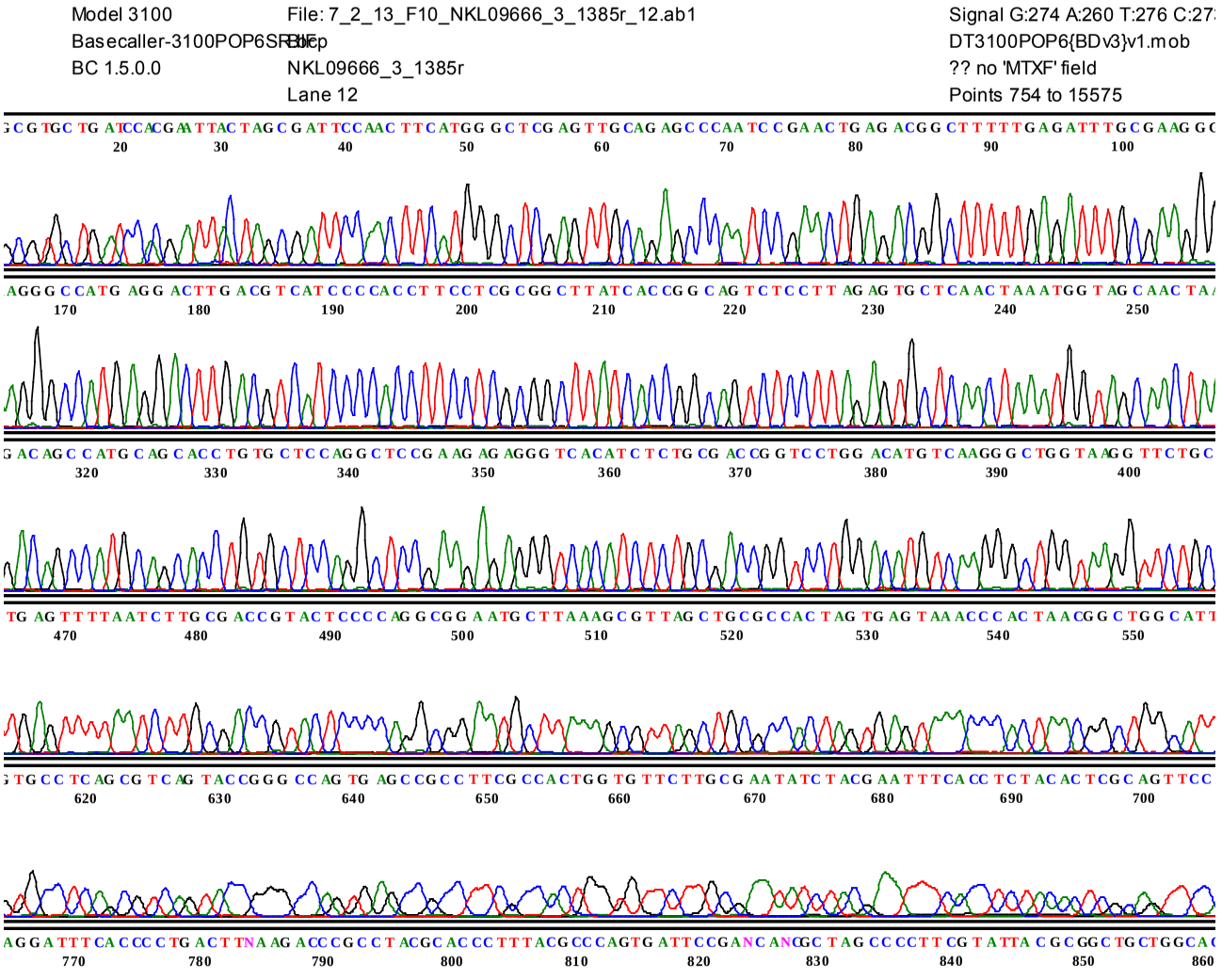

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 7_2_13_D09_NKL09666_2_343r_07.ab1
NKL09666_2_343r
Lane 7
Signal G:128 A:112 T:129 C:12
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 782 to 15575

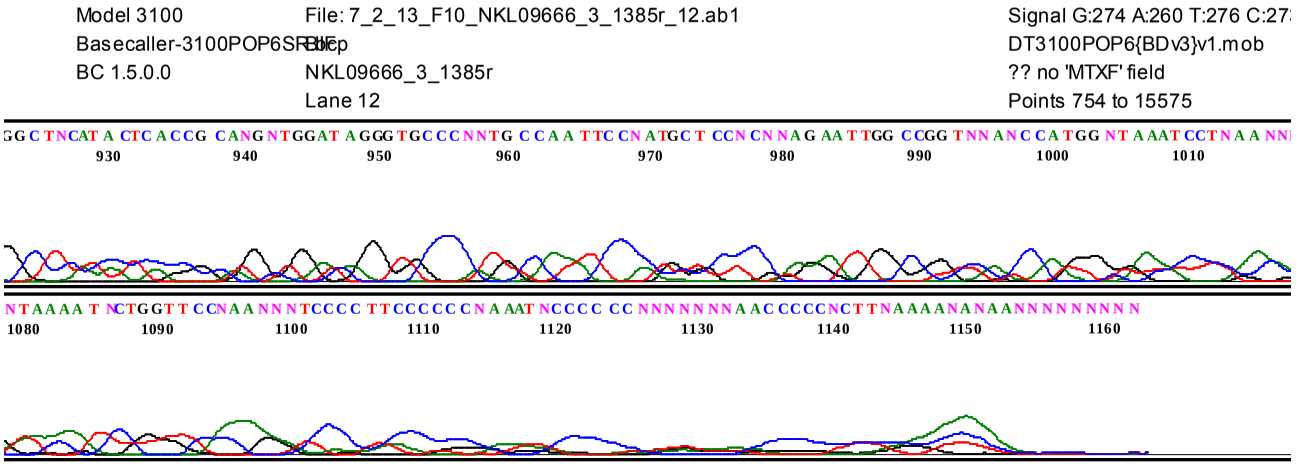

Model 3100
Basecaller-3100POP6SRBC
BC 1.5.0.0
File: 7_2_13_F09_NKL09666_2_519r_11.ab1
NKL09666_2_519r
Lane 11
Signal G:152 A:133 T:157 C:15
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1054 to 15575

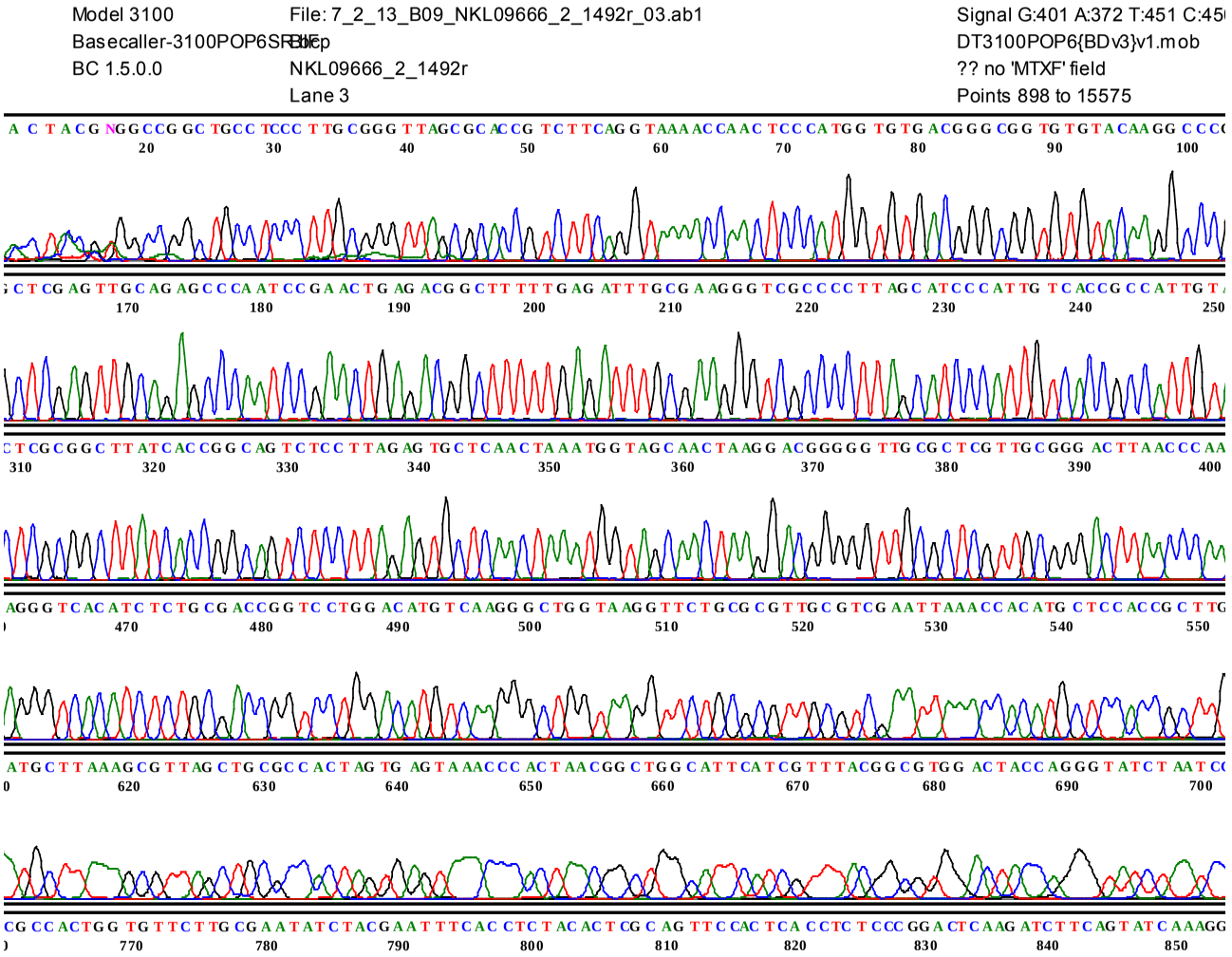

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 7_2_13_H09_NKL09666_2_787r_15.ab1
NKL09666_2_787r
Lane 15
Signal G:241 A:267 T:338 C:31
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 970 to 15575

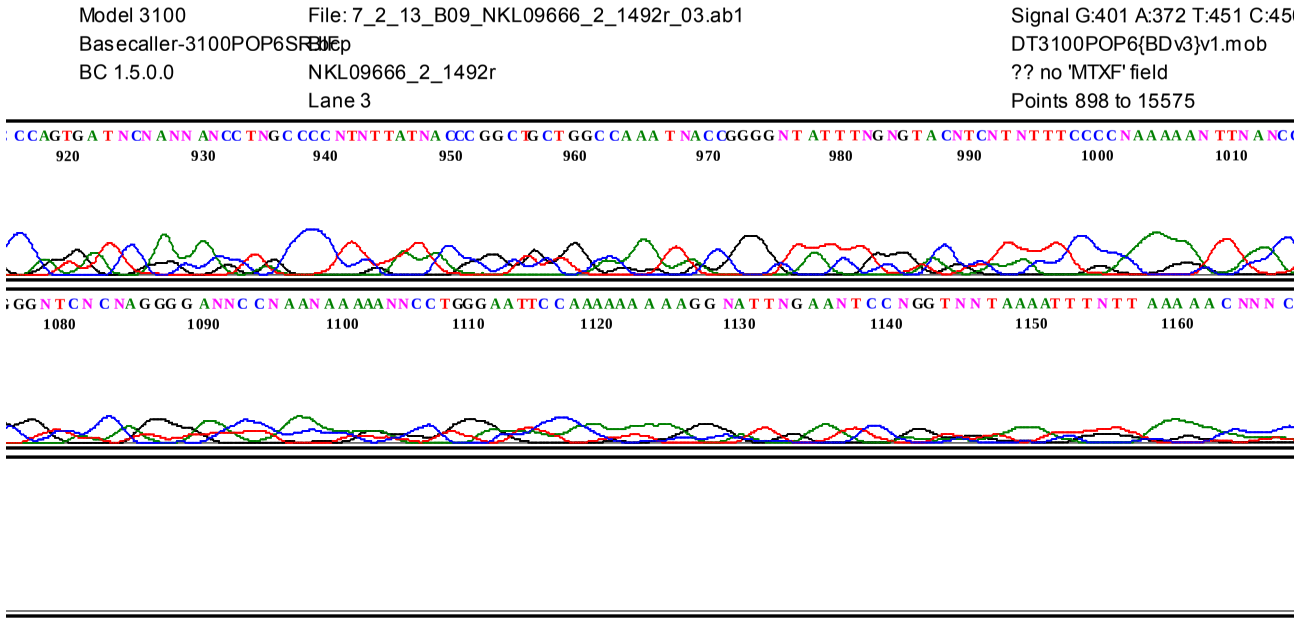

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 7_2_13_B10_NKL09666_3_907r_04.ab1
NKL09666_3_907r
Lane 4
Signal G:286 A:243 T:275 C:26
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1012 to 15575

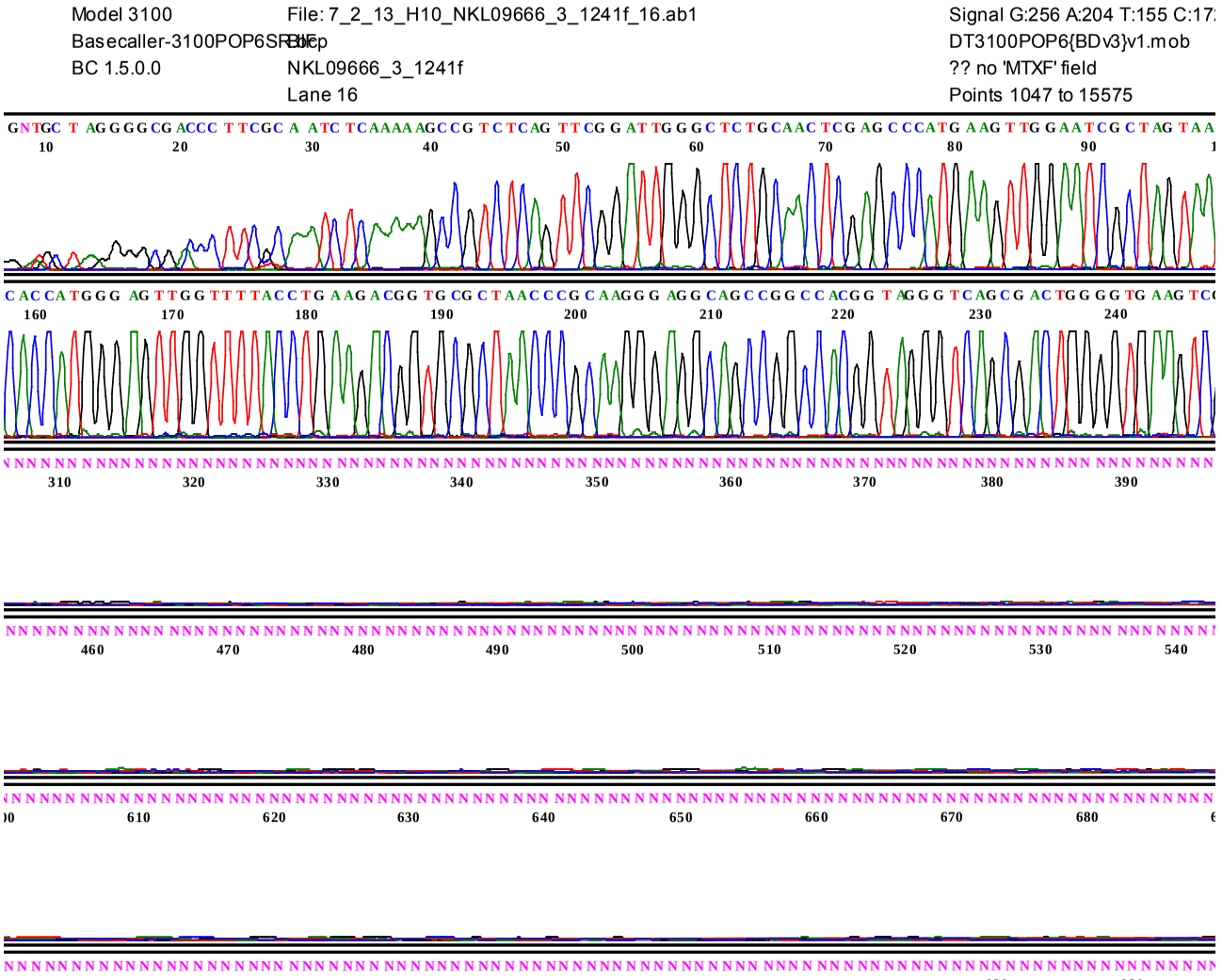

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 7_2_13_D10_NKL09666_3_1100r_08.ab1
NKL09666_3_1100r
Lane 8
Signal G:272 A:257 T:287 C:28
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 913 to 15575

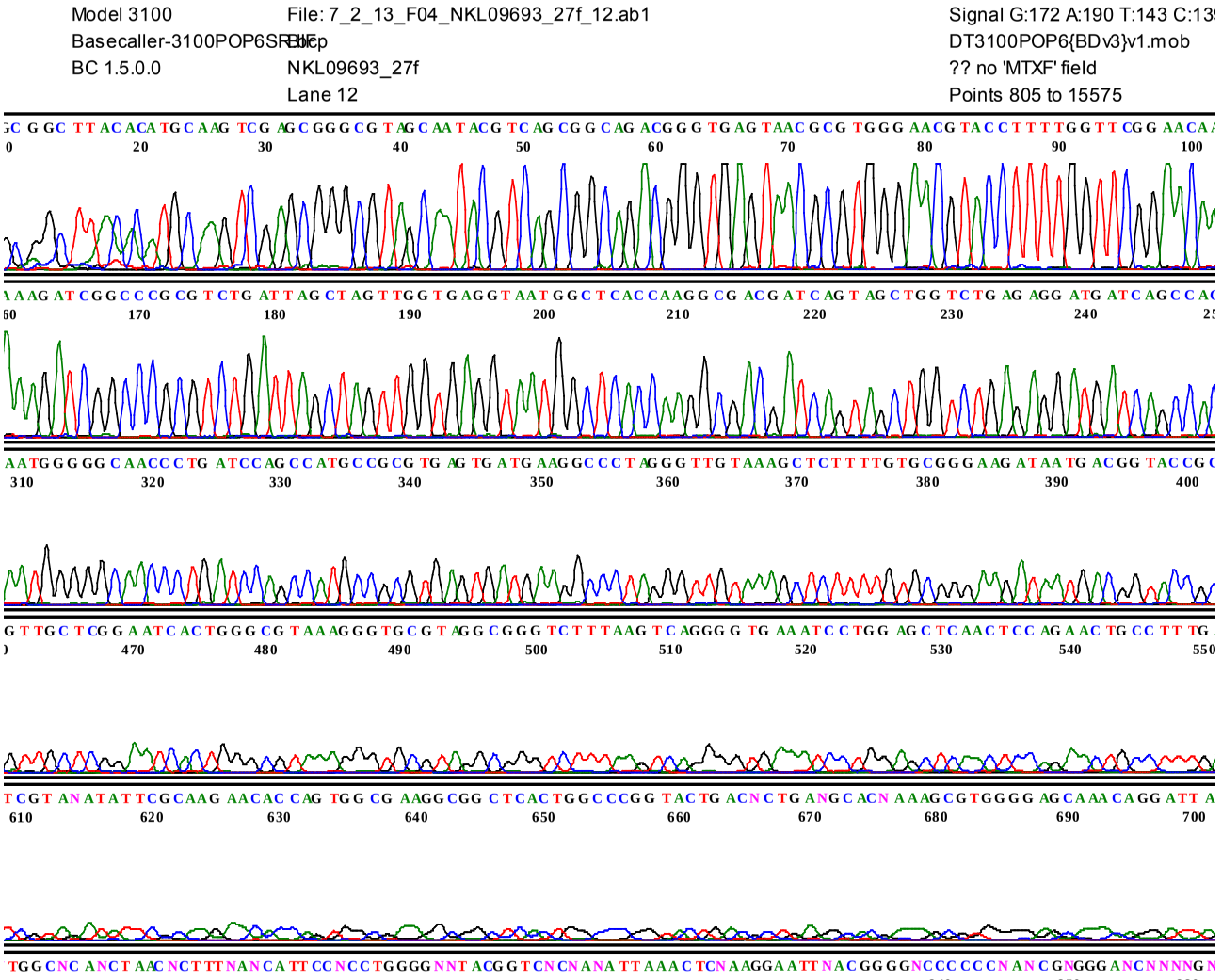

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 7_2_13_F10_NKL09666_3_1385r_12.ab1
NKL09666_3_1385r
Lane 12
Signal G:274 A:260 T:276 C:27
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 754 to 15575

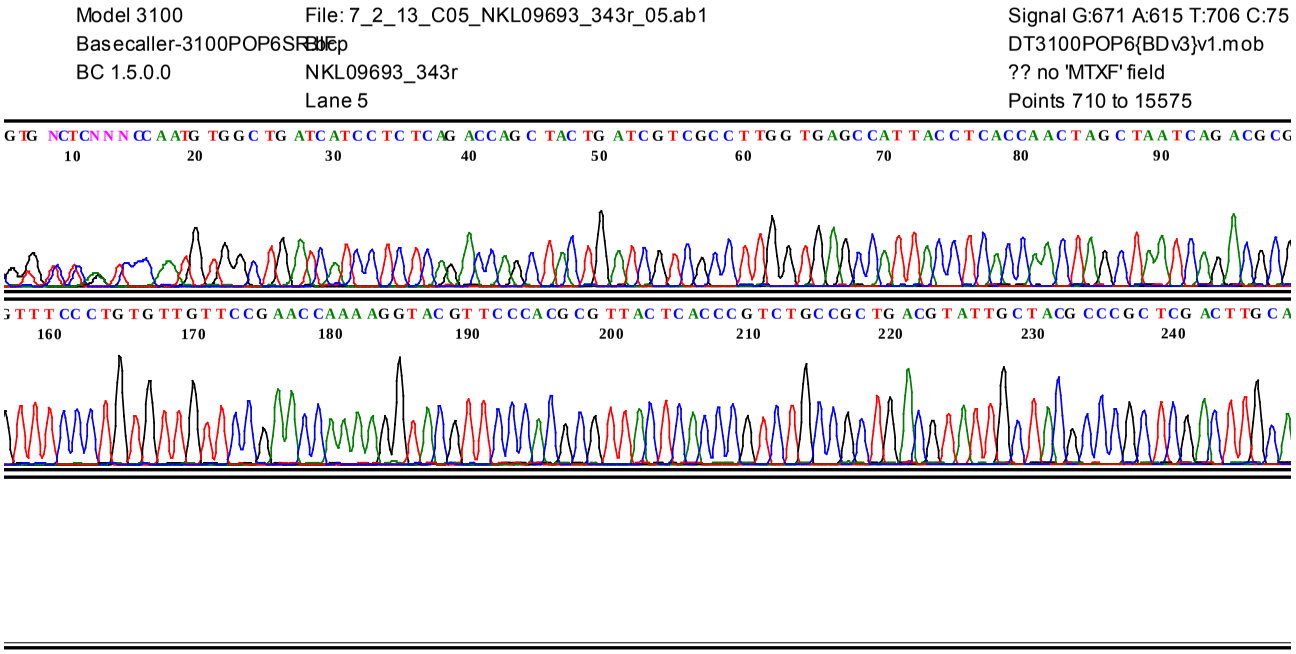

Model 3100
Basecaller-3100POP6SRBC
BC 1.5.0.0
File: 7_2_13_F10_NKL09666_3_1385r_12.ab1
NKL09666_3_1385r
Lane 12
Signal G:274 A:260 T:276 C:27
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 754 to 15575
930
940
950
960
970
980
990
1000
1010
1080
1090
1100
1110
1120
1130
1140
1150
1160

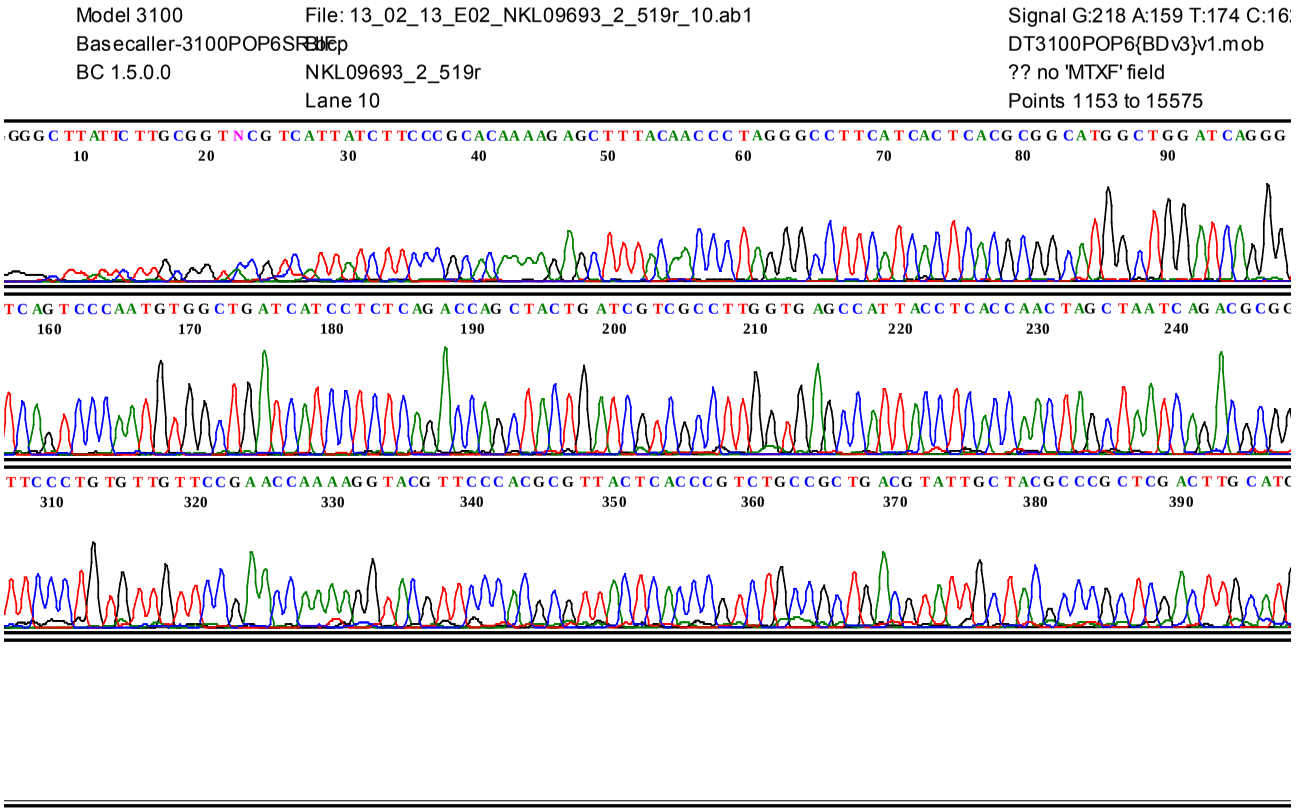

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 7_2_13_B09_NKL09666_2_1492r_03.ab1
NKL09666_2_1492r
Lane 3
Signal G:401 A:372 T:451 C:45
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 898 to 15575

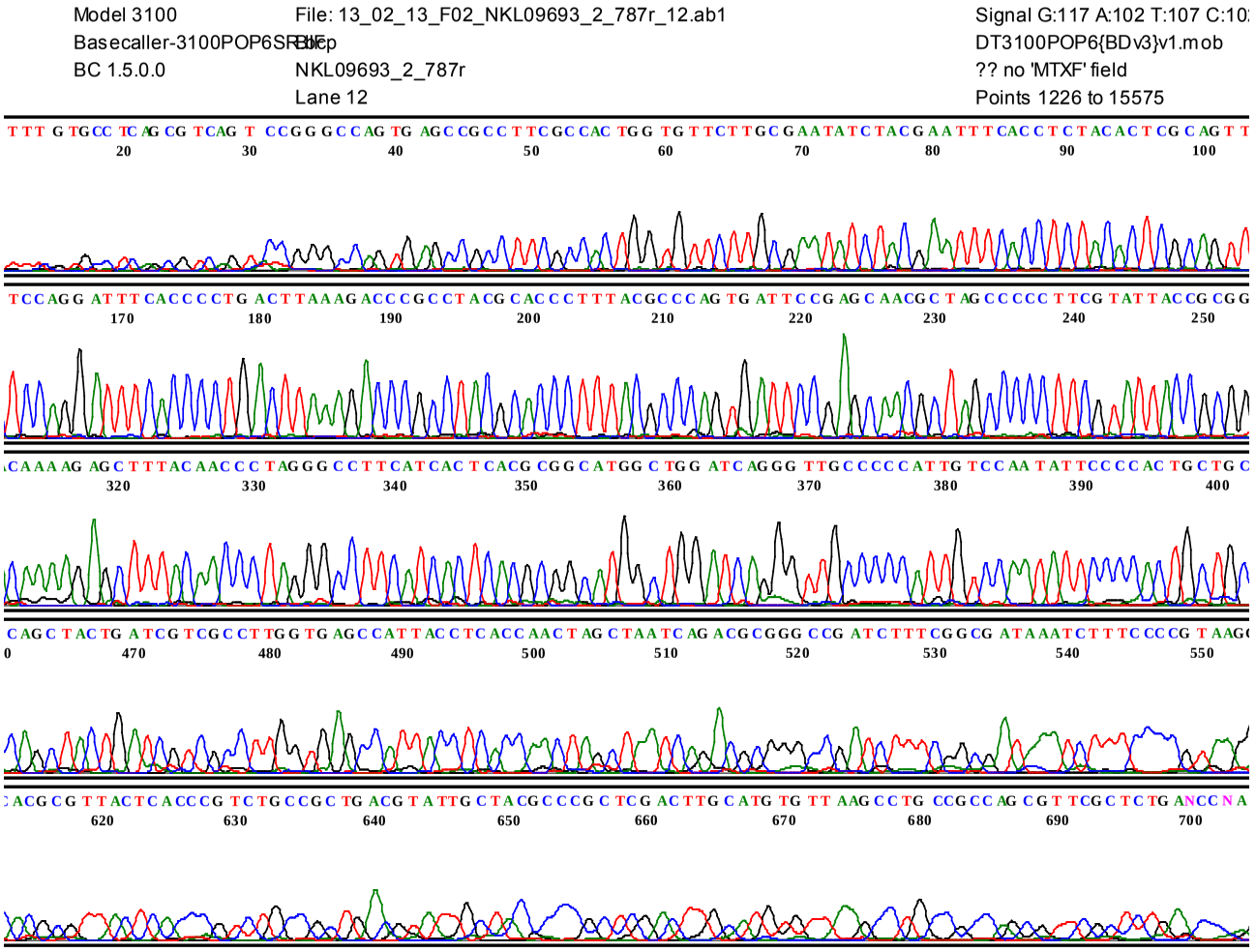

Model 3100
File: 7_2_13_B09_NKL09666_2_1492r_03.ab1
Signal G:401 A:372 T:451 C:45
Basecaller-3100POP6SRBDep
DT3100POP6{BDv3}v1.mob
BC 1.5.0.0
NKL09666_2_1492r
?? no 'MTXF' field
Lane 3
Points 898 to 15575
920
930
940
950
960
970
980
990
1000
1010
1080
1090
1100
1110
1120
1130
1140
1150
1160

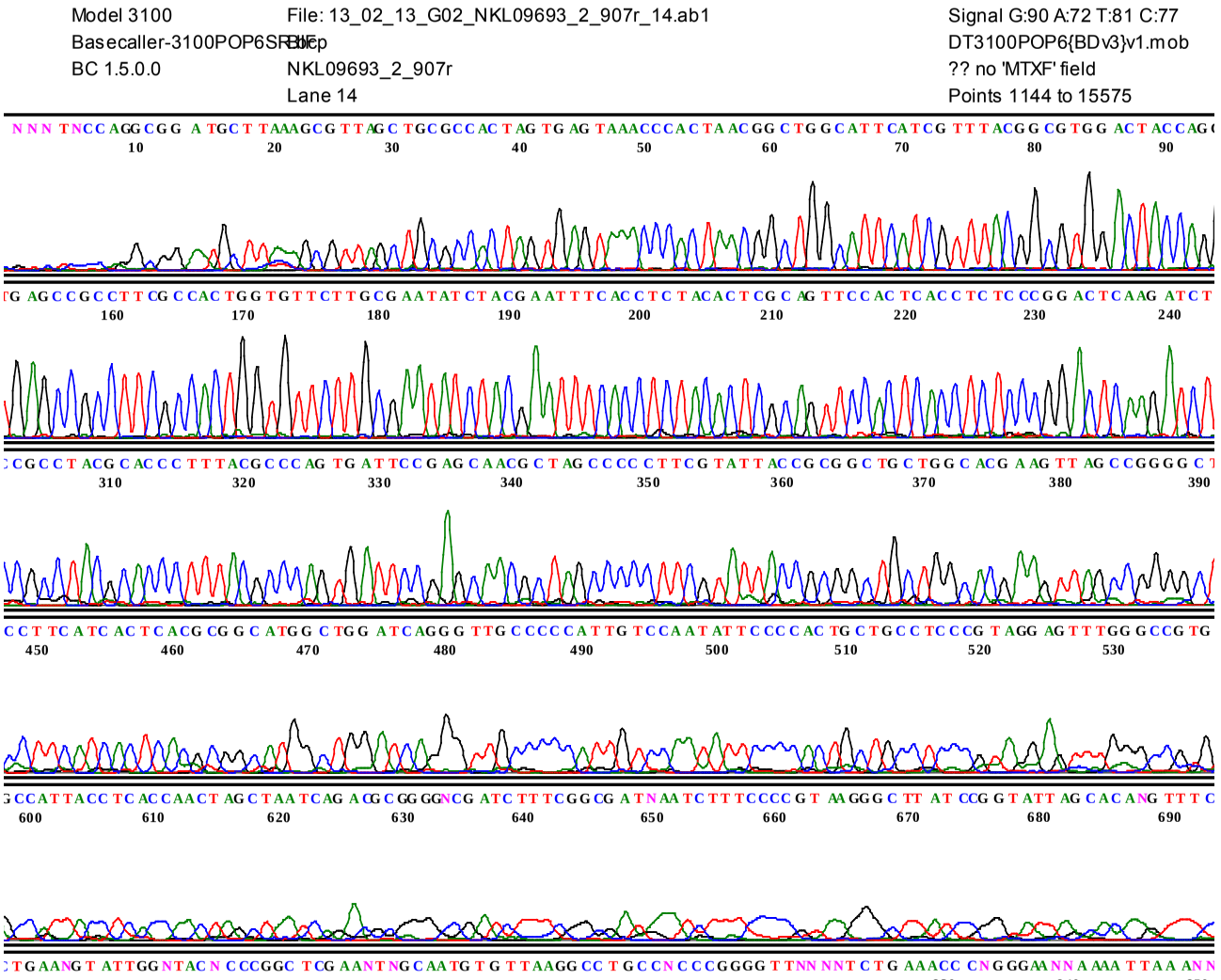

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 7_2_13_H10_NKL09666_3_1241f_16.ab1
Ep
NKL09666_3_1241f
Lane 16
Signal G:256 A:204 T:155 C:17
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1047 to 15575

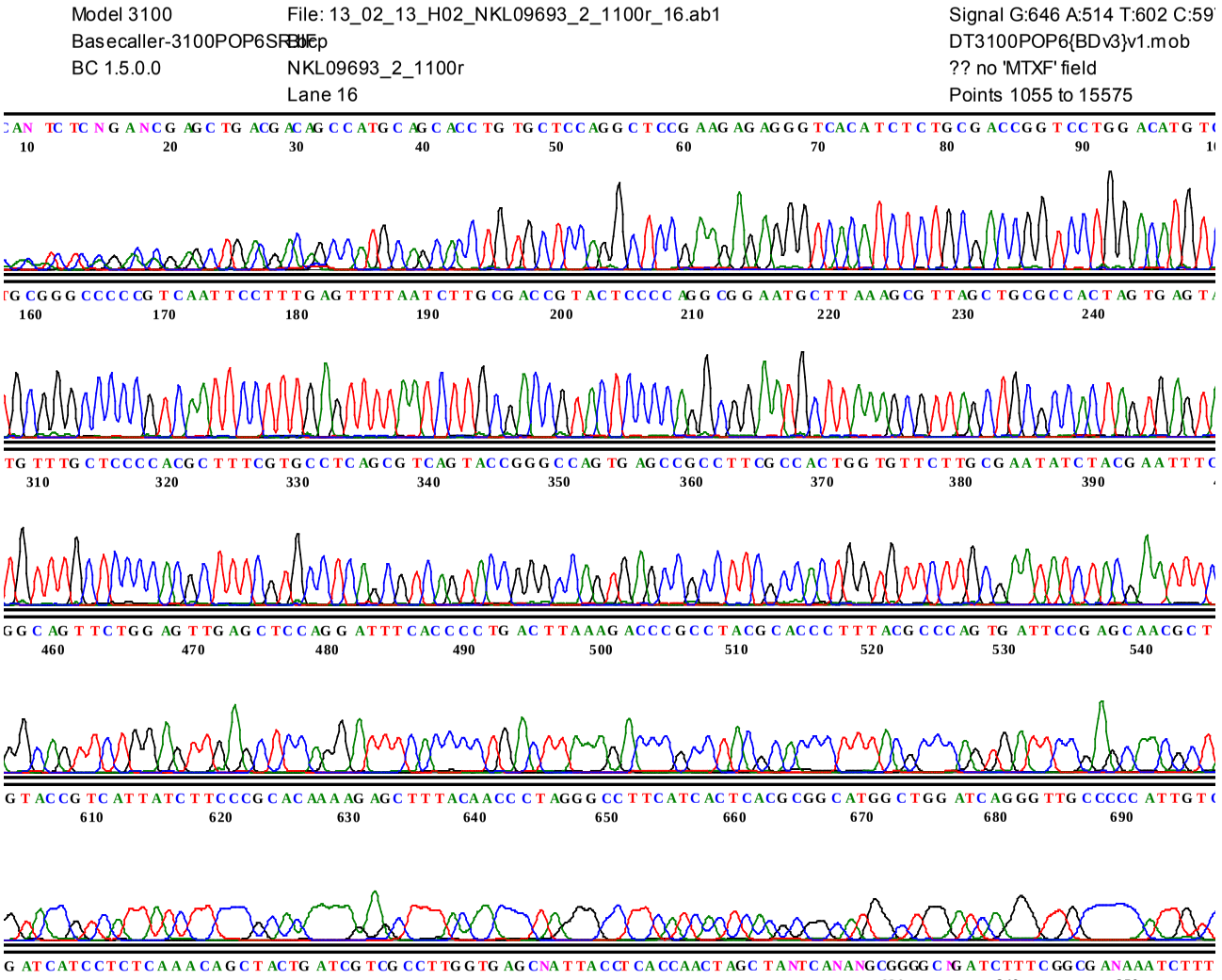

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 7_2_13_F04_NKL09693_27f_12.ab1
NKL09693_27f
Lane 12
Signal G:172 A:190 T:143 C:13
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 805 to 15575

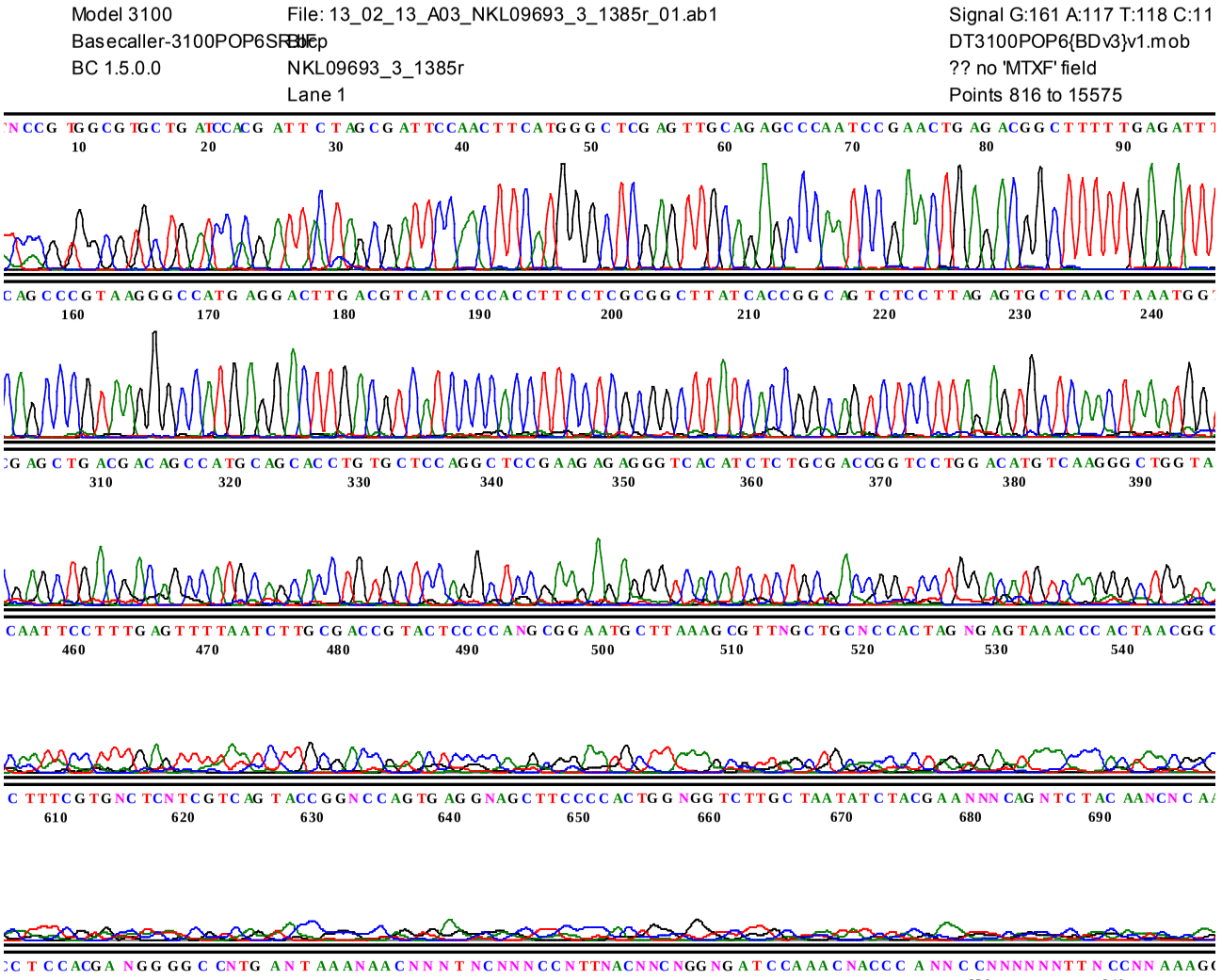

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 7_2_13_C05_NKL09693_343r_05.ab1
NKL09693_343r
Lane 5
Signal G:671 A:615 T:706 C:75
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 710 to 15575

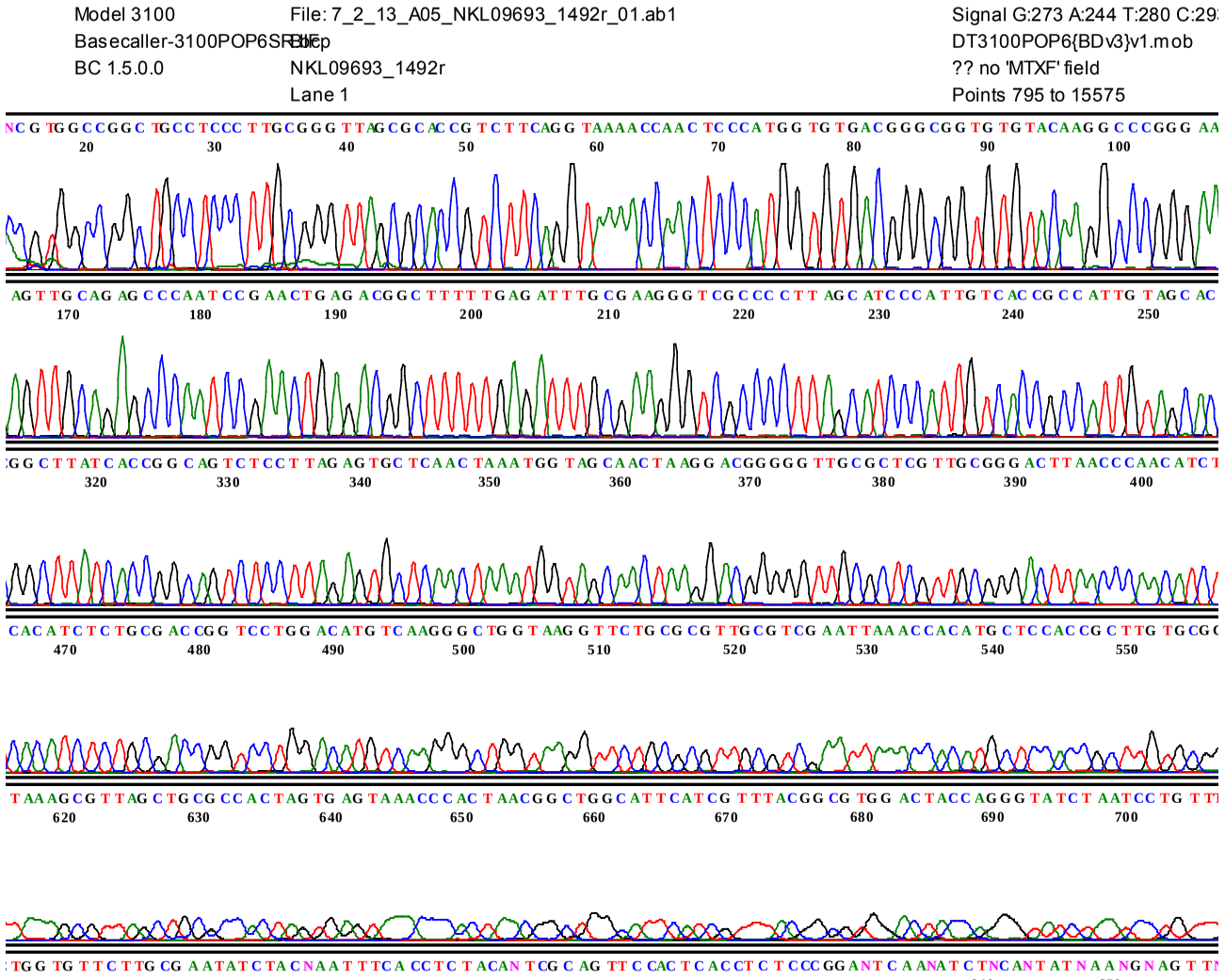

Model 3100
Basecaller-3100POP6SRBDF
BC 1.5.0.0
File: 13_02_13_E02_NKL09693_2_519r_10.ab1
Ep
NKL09693_2_519r
Lane 10
Signal G:218 A:159 T:174 C:16
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1153 to 15575

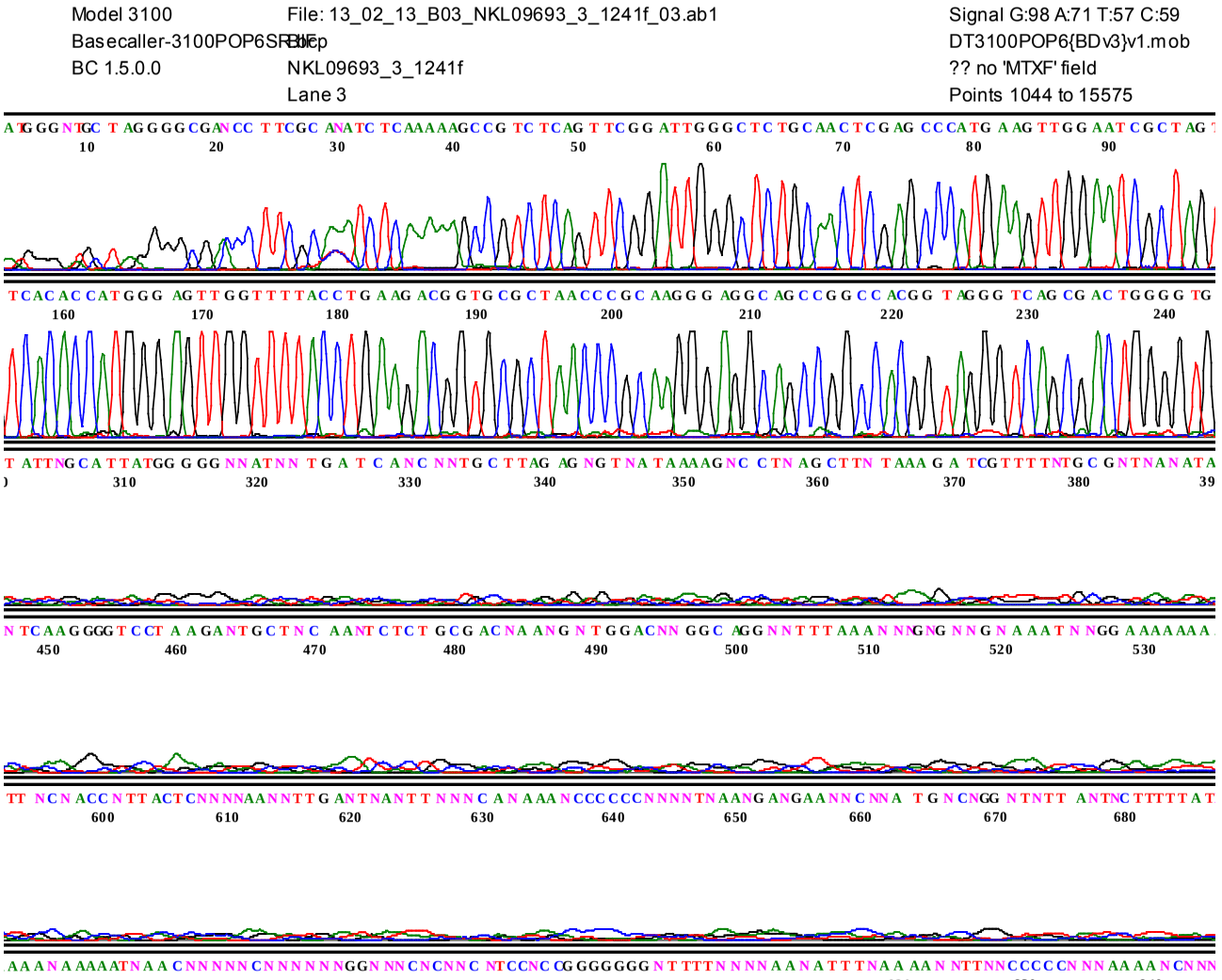

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 13_02_13_F02_NKL09693_2_787r_12.ab1
Ebcp
NKL09693_2_787r
Lane 12
Signal G:117 A:102 T:107 C:10
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1226 to 15575

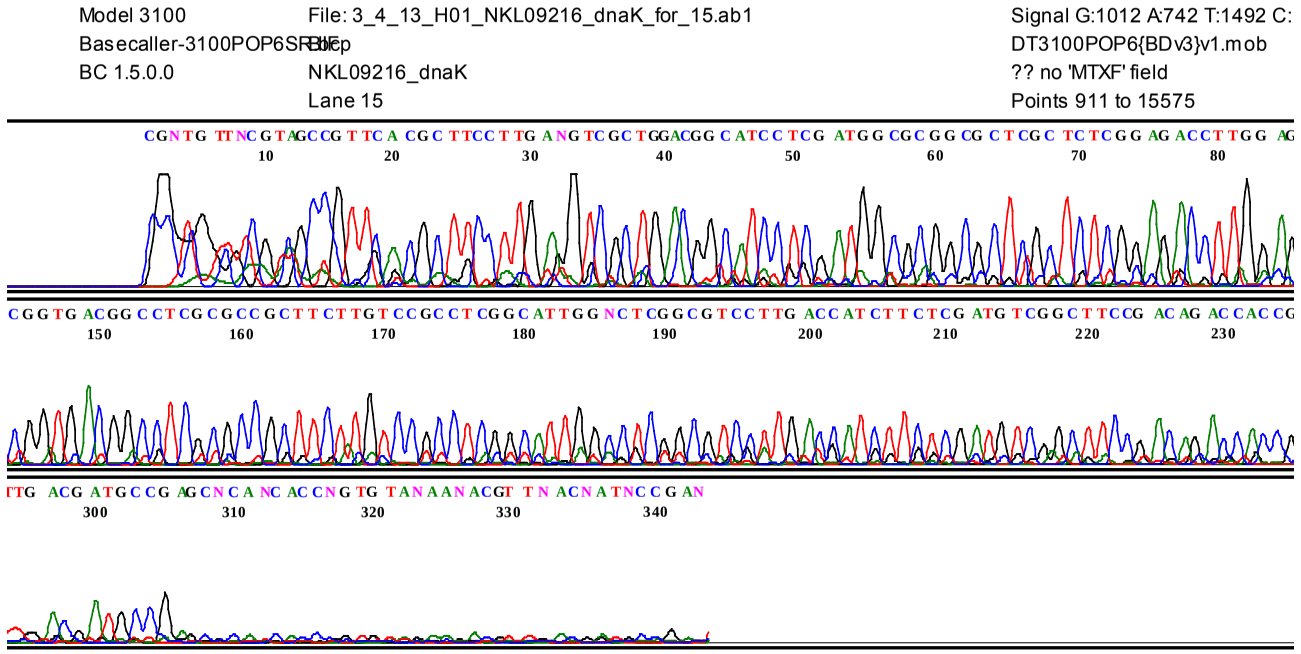

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 13_02_13_G02_NKL09693_2_907r_14.ab1
NKL09693_2_907r
Lane 14
Signal G:90 A:72 T:81 C:77
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1144 to 15575

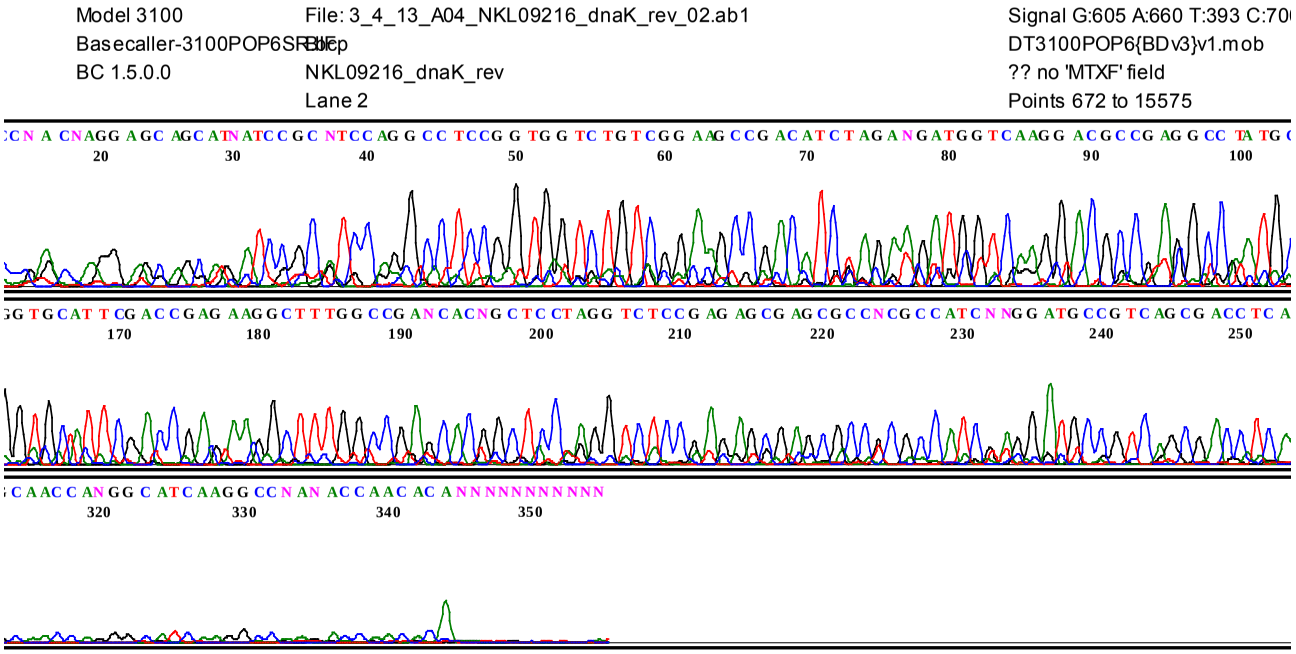

Model 3100
File: 13_02_13_H02_NKL09693_2_1100r_16.ab1
Signal G:646 A:514 T:602 C:59
Basecaller-3100POP6SRBDEp
DT3100POP6{BDv3}v1.mob
BC 1.5.0.0
NKL09693_2_1100r
?? no 'MTXF' field
Lane 16
Points 1055 to 15575

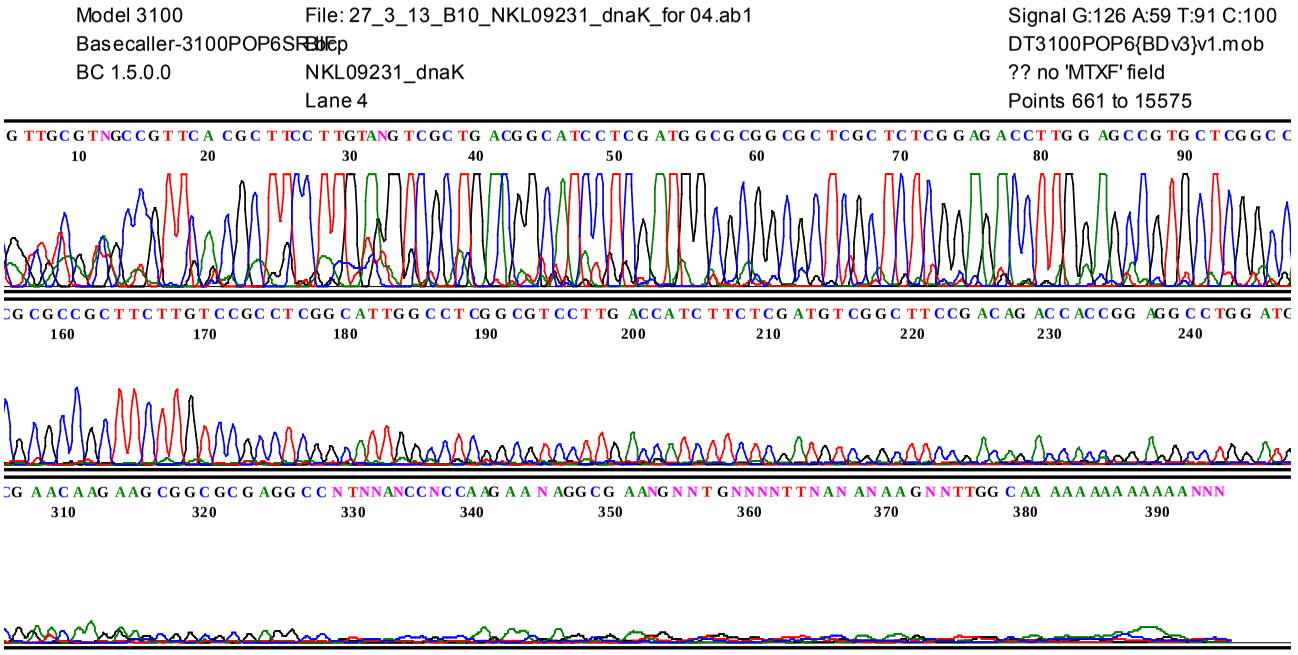

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 13_02_13_A03_NKL09693_3_1385r_01.ab1
NKL09693_3_1385r
Lane 1
Signal G:161 A:117 T:118 C:11
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 816 to 15575

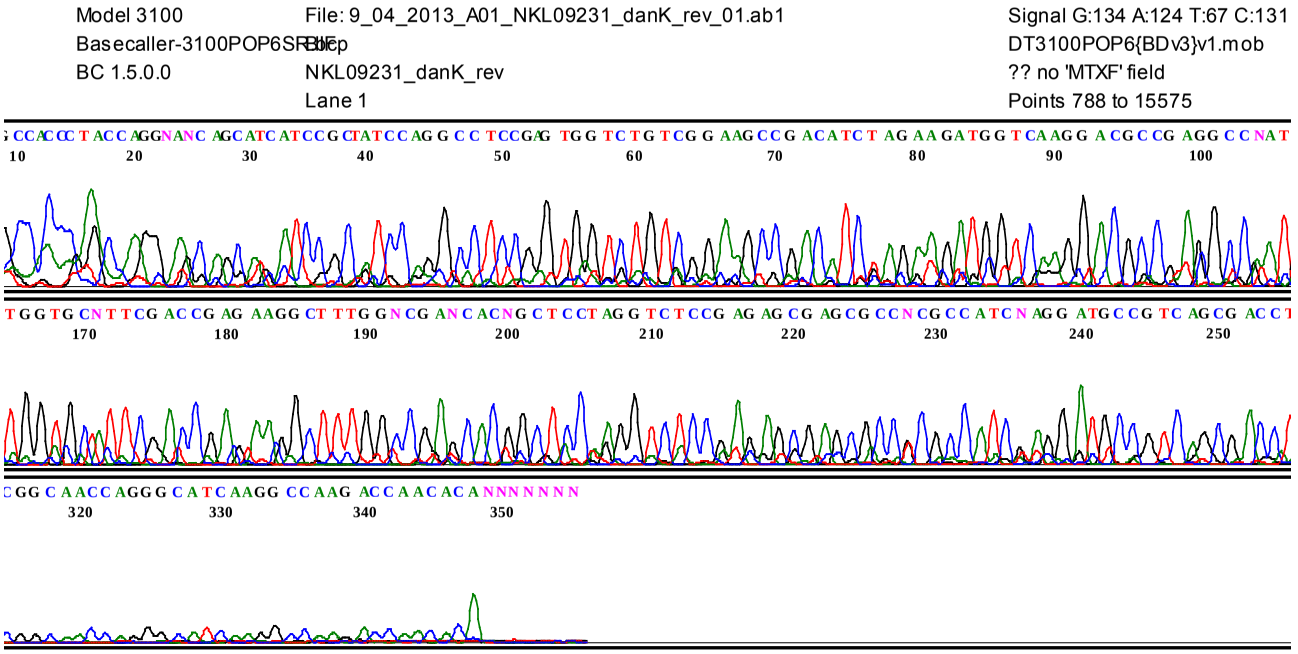

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 7_2_13_A05_NKL09693_1492r_01.ab1
NKL09693_1492r
Lane 1
Signal G:273 A:244 T:280 C:29
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 795 to 15575

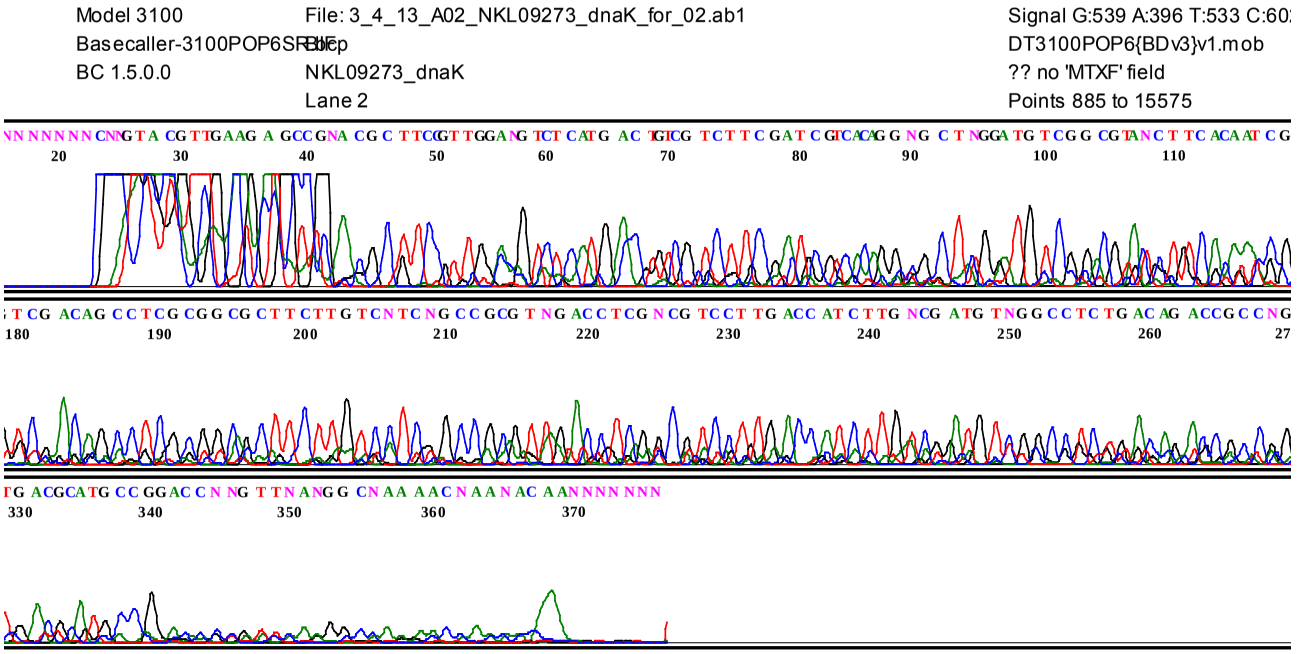

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 13_02_13_B03_NKL09693_3_1241f_03.ab1
NKL09693_3_1241f
Lane 3
Signal G:98 A:71 T:57 C:59
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1044 to 15575

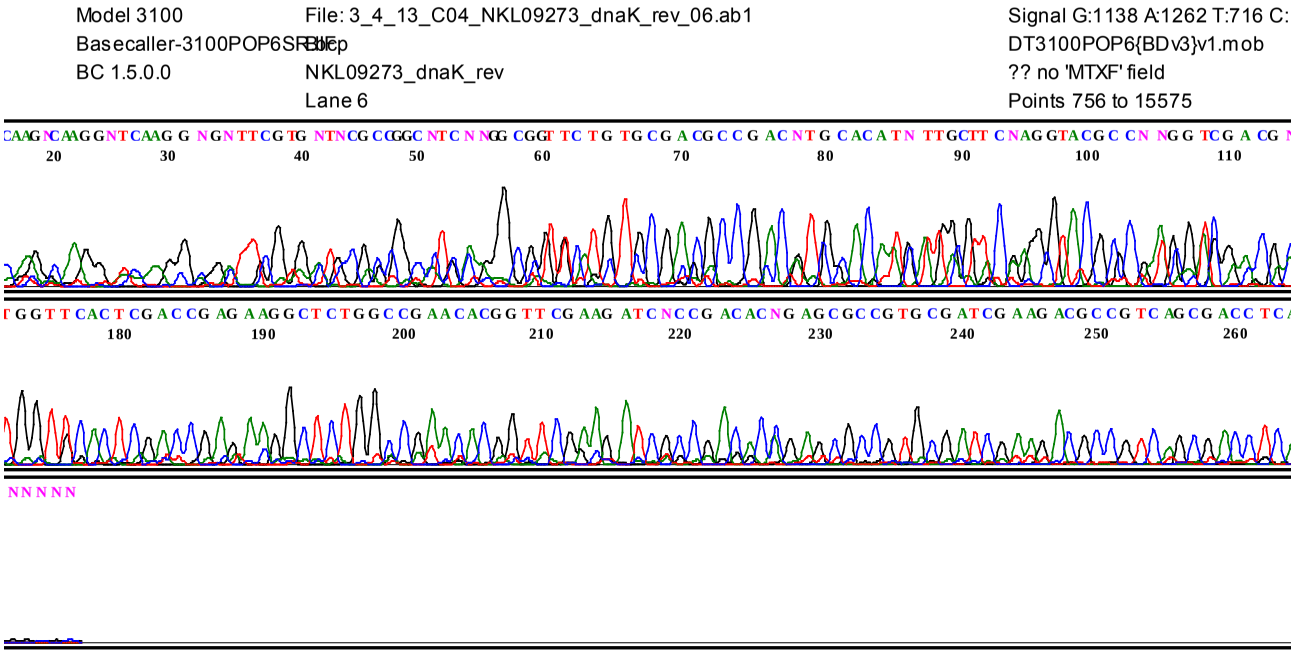

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 3_4_13_H01_NKL09216_dnaK_for_15.ab1
NKL09216_dnaK
Lane 15
Signal G:1012 A:742 T:1492 C:
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 911 to 15575
CGNTG TTNCGTAGCCG TTCA CGC TTCCTTG ANG TCGCTGGACGG CATCC TCG ATGG CGC GG CG CTCGC TC TCGG AG ACCTTGG AG
10 20 30 40 50 60 70 80
CGGTG ACGG CCTCG CGCC GCTT CTTG TCCGCCT CGGCATTGG NCTCGGCG TCCTTG ACCATCTTCTCG ATG TCGGCTTCCG AC AG AC CAC CG
150 160 170 180 190 200 210 220 230
TTG ACG ATGCCG AGCN CA NC AC CNG TG TANAAN ACGT TN ACN ATNCCG AN
300 310 320 330 340

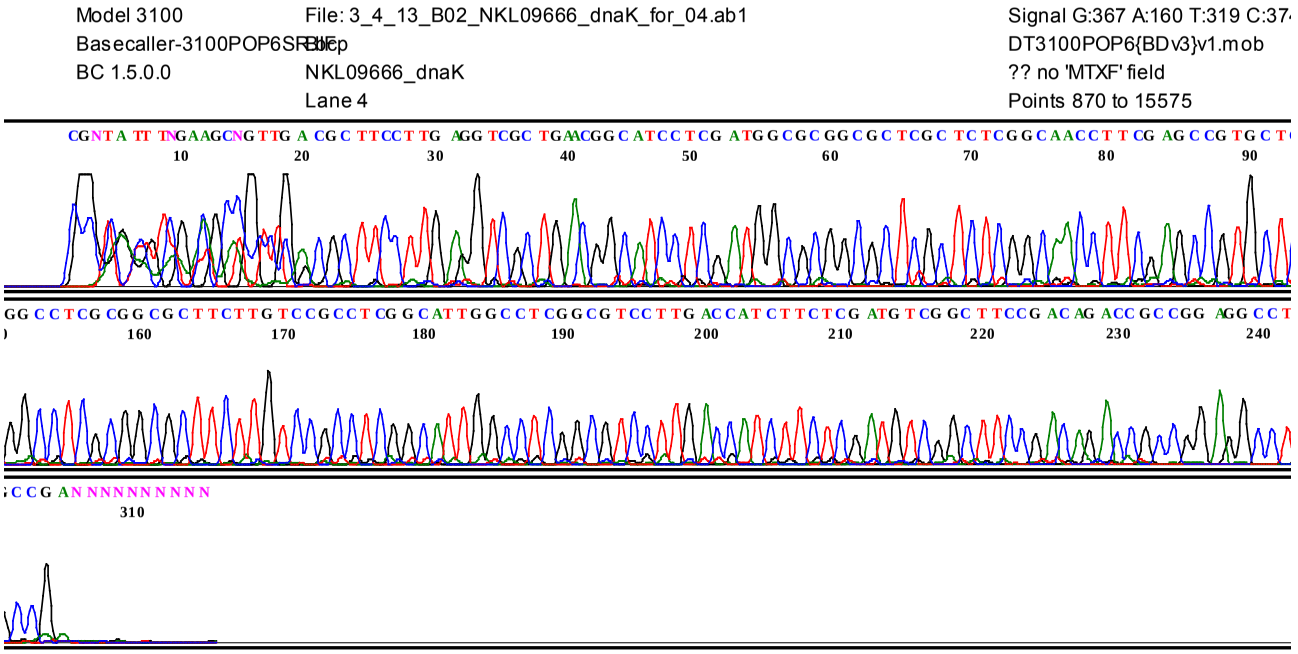

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 3_4_13_A04_NKL09216_dnaK_rev_02.ab1
NKL09216_dnaK_rev
Lane 2
Signal G:605 A:660 T:393 C:70
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 672 to 15575

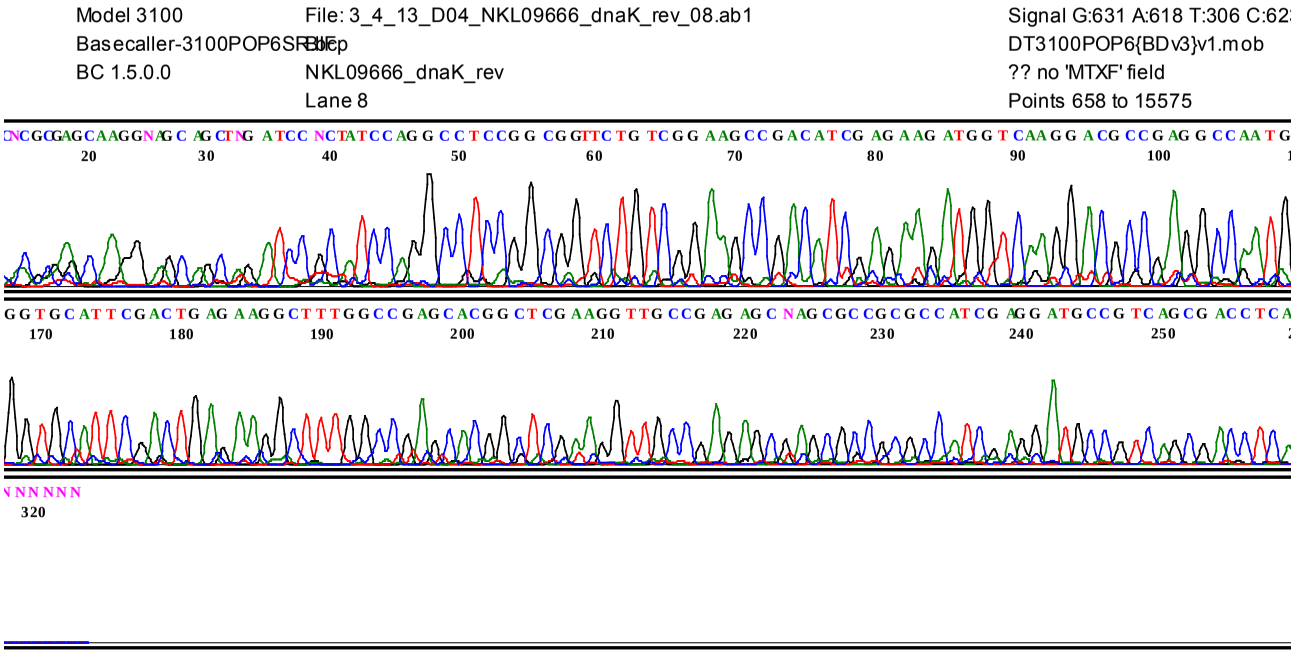

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 27_3_13_B10_NKL09231_dnaK_for 04.ab1
NKL09231_dnaK
Lane 4
Signal G:126 A:59 T:91 C:100
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 661 to 15575

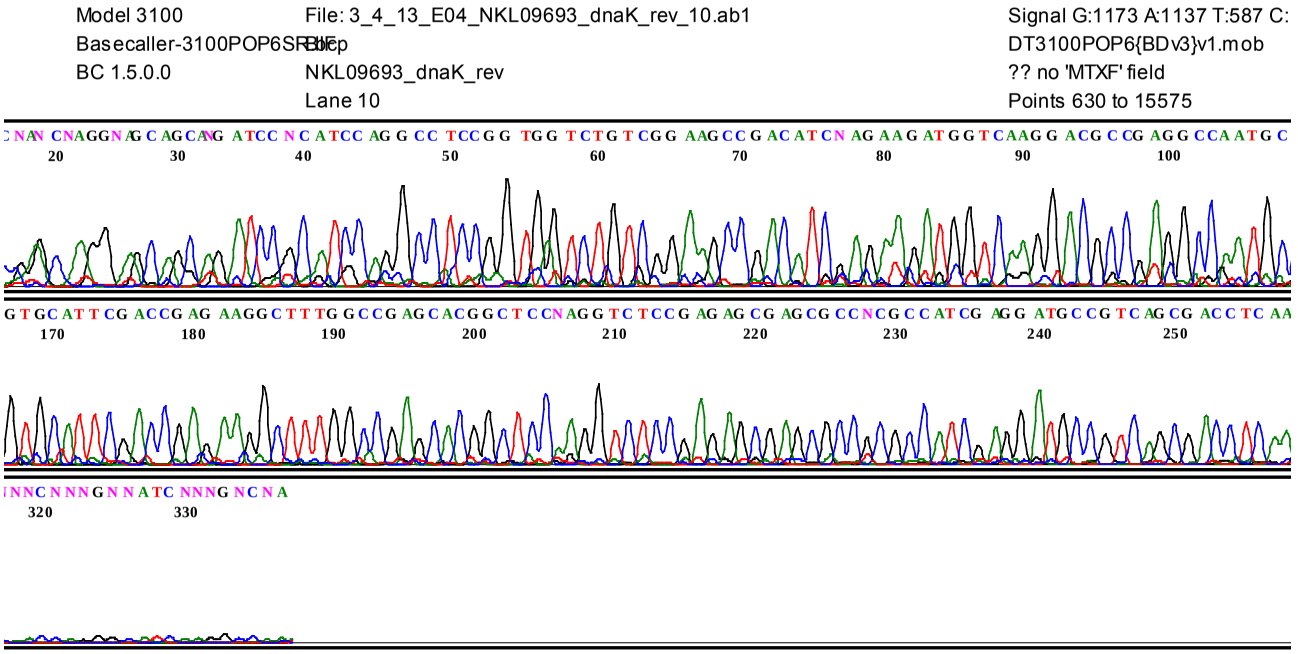

Model 3100
Basecaller-3100POP6SRBC
BC 1.5.0.0
File: 9_04_2013_A01_NKL09231_danK_rev_01.ab1
NKL09231_danK_rev
Lane 1
Signal G:134 A:124 T:67 C:131
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 788 to 15575

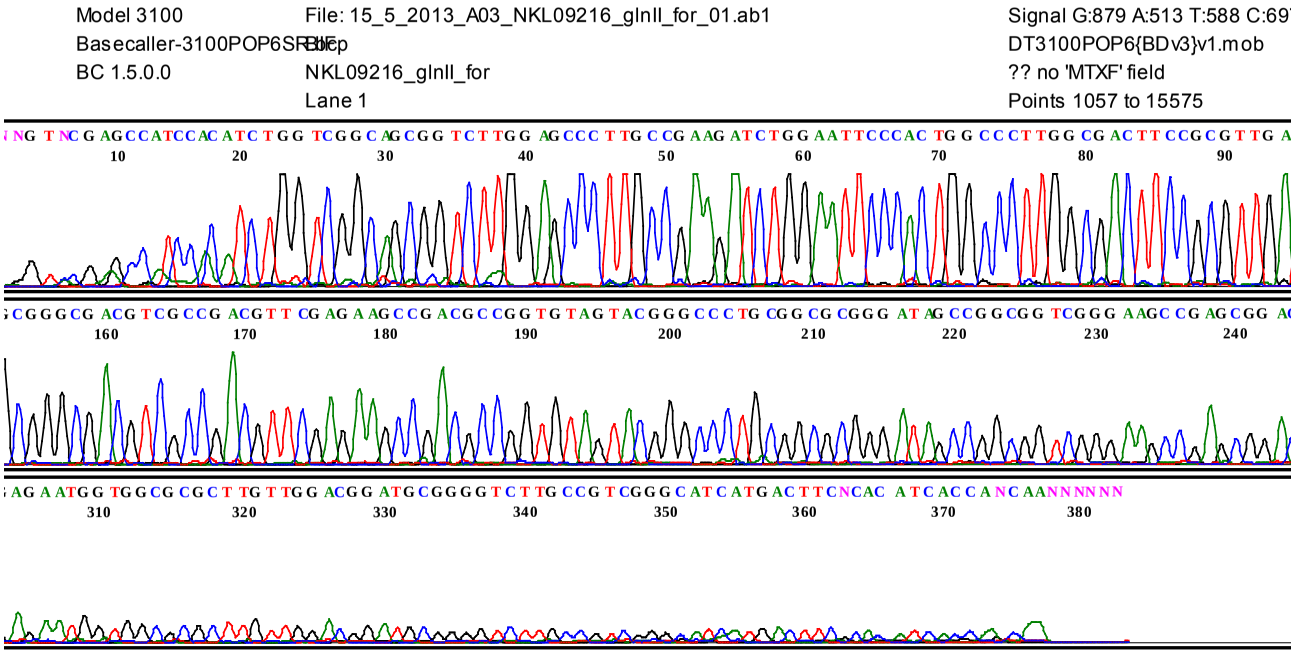

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 3_4_13_A02_NKL09273_dnaK_for_02.ab1
NKL09273_dnaK
Lane 2
Signal G:539 A:396 T:533 C:60
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 885 to 15575

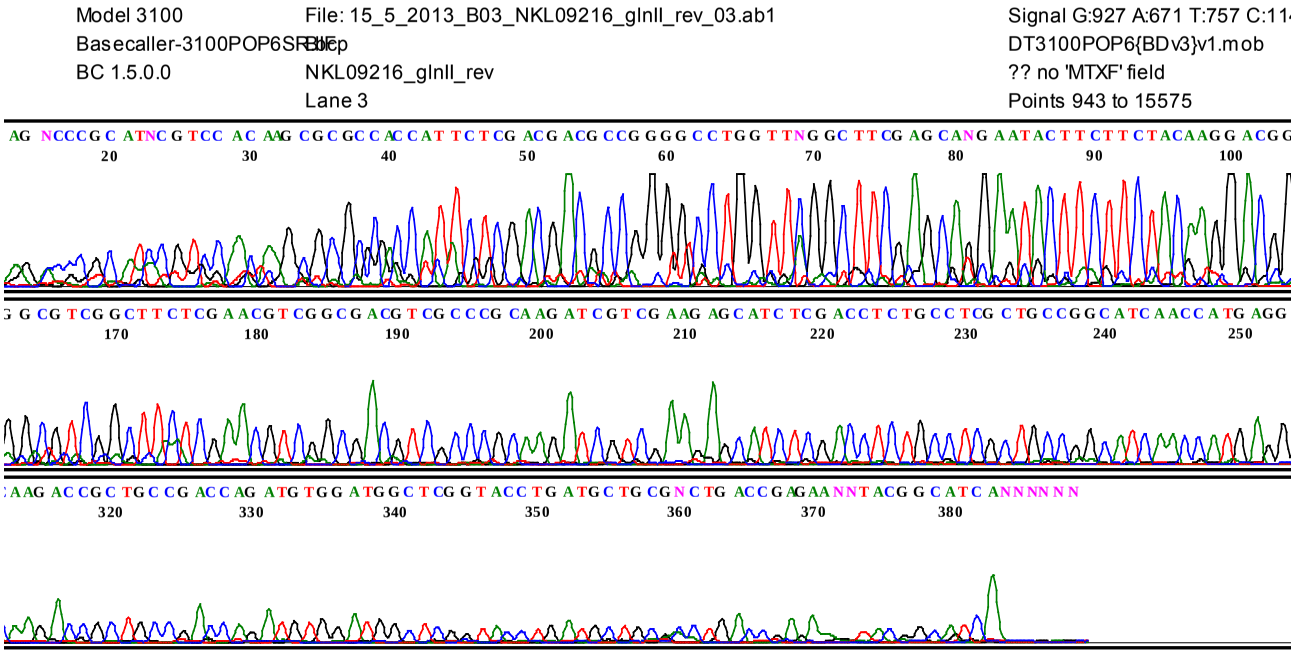

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 3_4_13_C04_NKL09273_dnaK_rev_06.ab1
NKL09273_dnaK_rev
Lane 6
Signal G:1138 A:1262 T:716 C:
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 756 to 15575

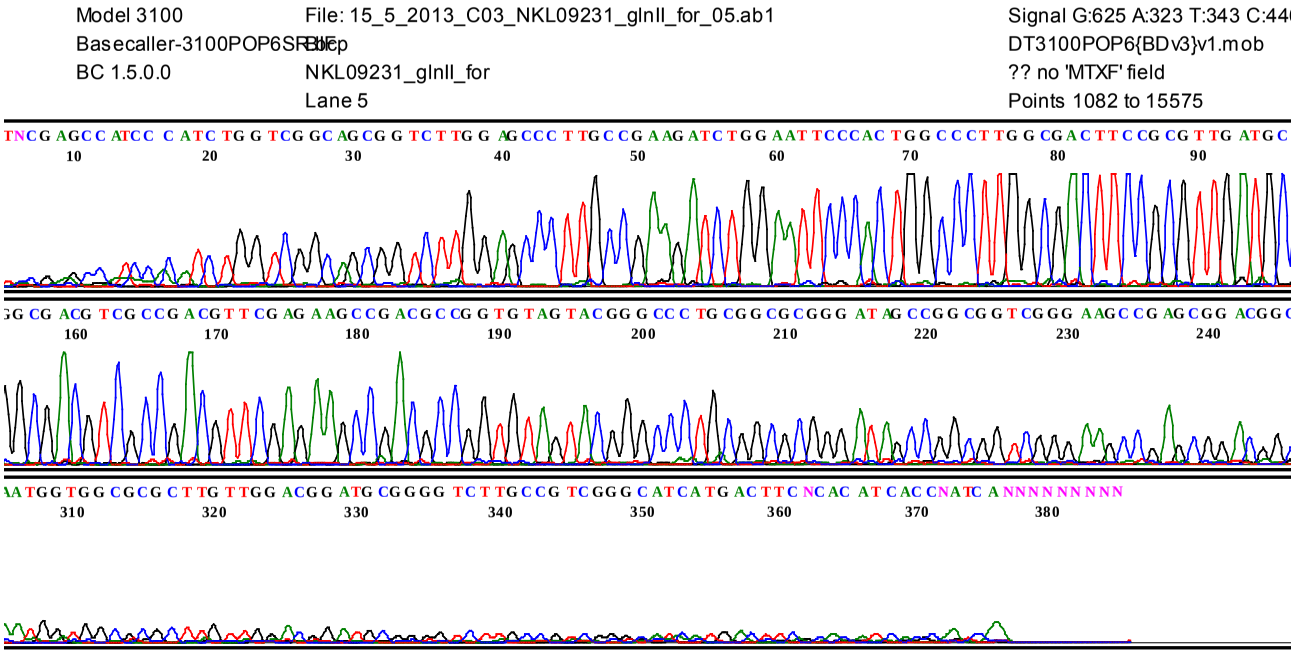

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 3_4_13_B02_NKL09666_dnaK_for_04.ab1
NKL09666_dnaK
Lane 4
Signal G:367 A:160 T:319 C:37
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 870 to 15575

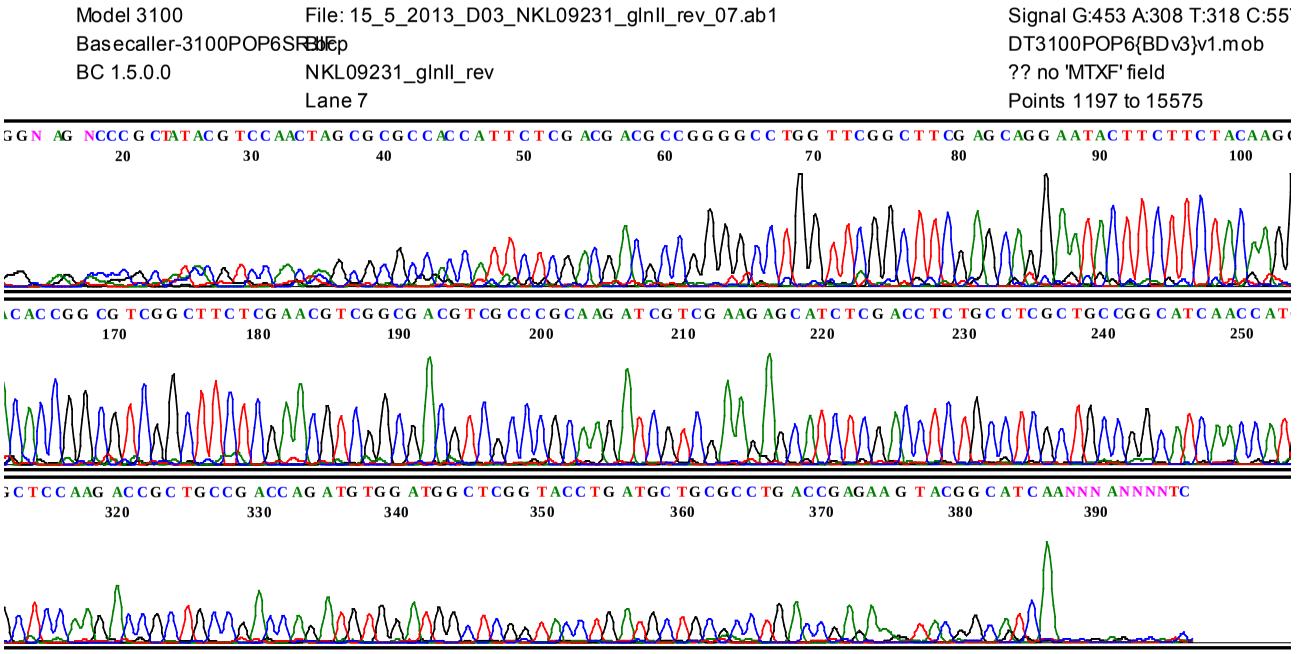

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 3_4_13_D04_NKL09666_dnaK_rev_08.ab1
NKL09666_dnaK_rev
Lane 8
Signal G:631 A:618 T:306 C:62
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 658 to 15575

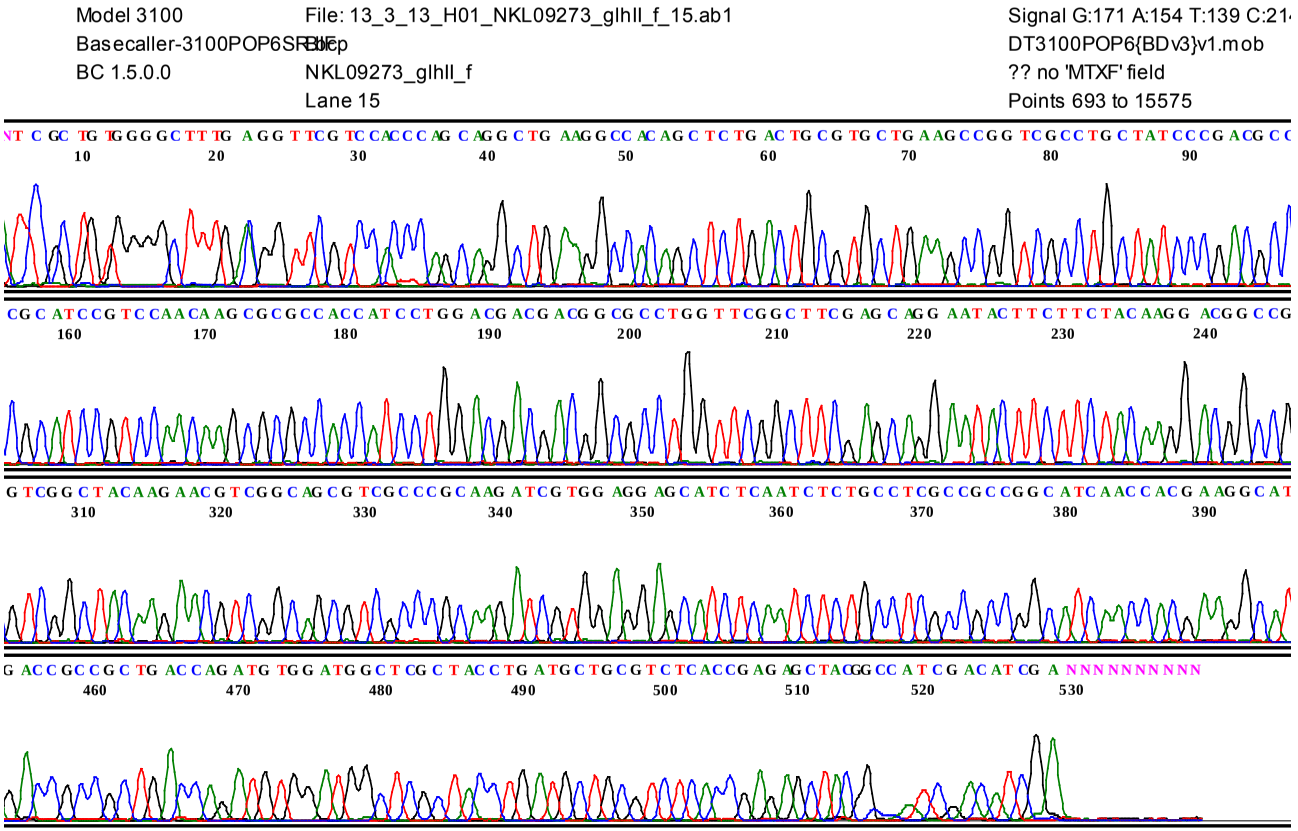

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 3_4_13_E04_NKL09693_dnaK_rev_10.ab1
NKL09693_dnaK_rev
Lane 10
Signal G:1173 A:1137 T:587 C:
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 630 to 15575

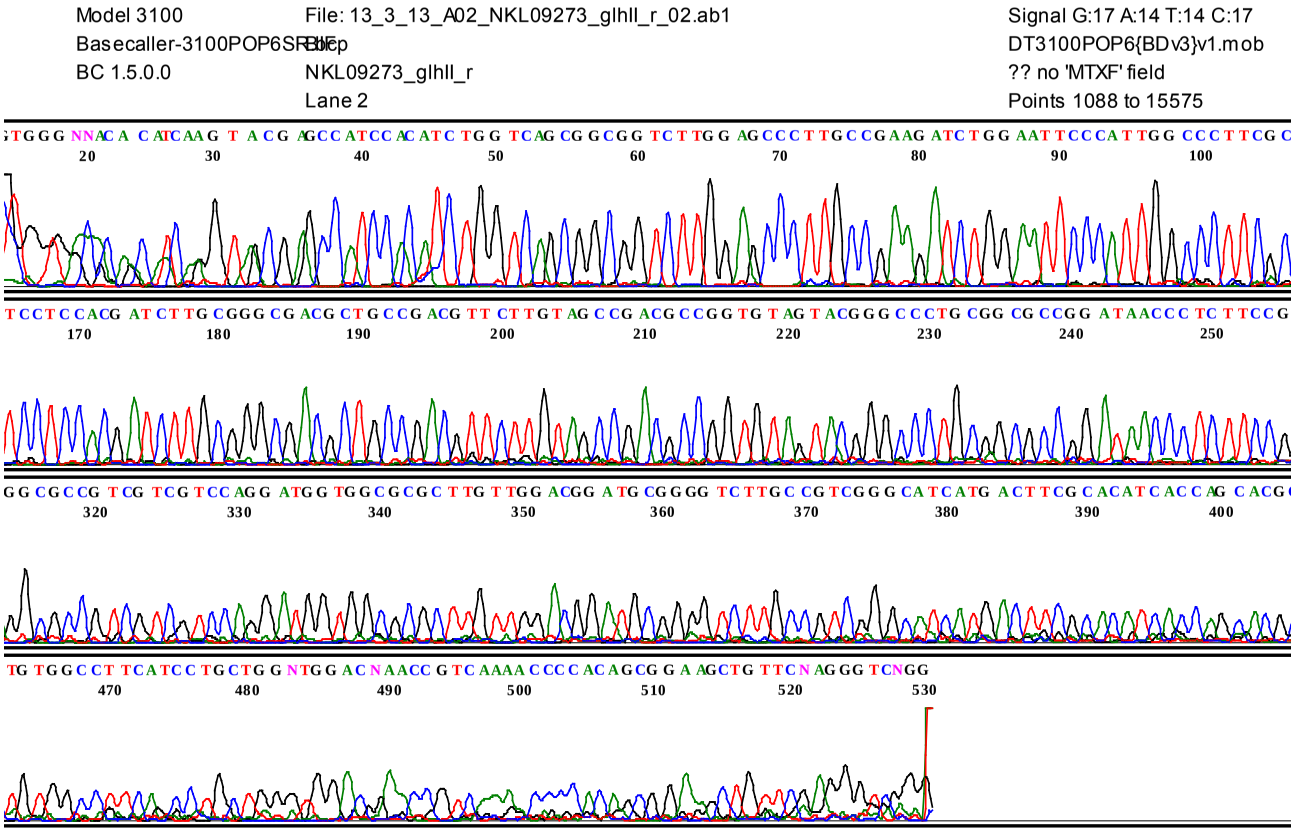

Model 3100
Basecaller-3100POP6SRBDF
BC 1.5.0.0
File: 15_5_2013_A03_NKL09216_glnII_for_01.ab1
Ebcp
NKL09216_glnII_for
Lane 1
Signal G:879 A:513 T:588 C:69
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1057 to 15575

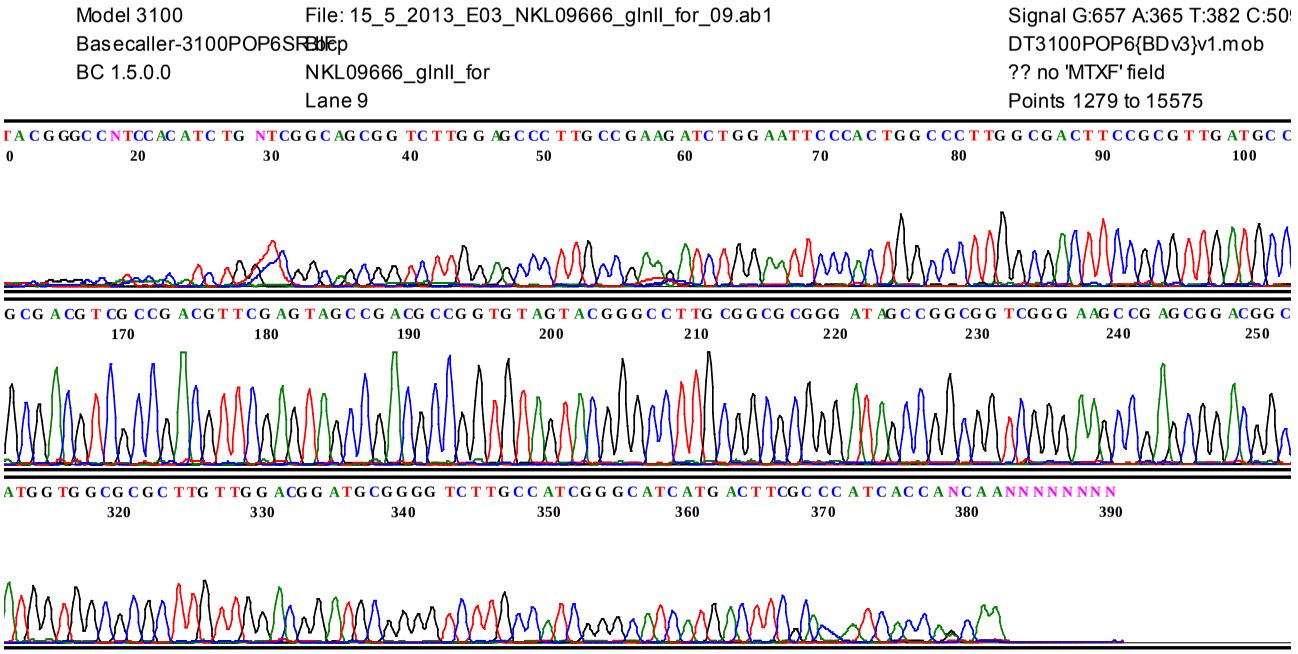

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 15_5_2013_B03_NKL09216_glnII_rev_03.ab1
NKL09216_glnII_rev
Lane 3
Signal G:927 A:671 T:757 C:11
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 943 to 15575

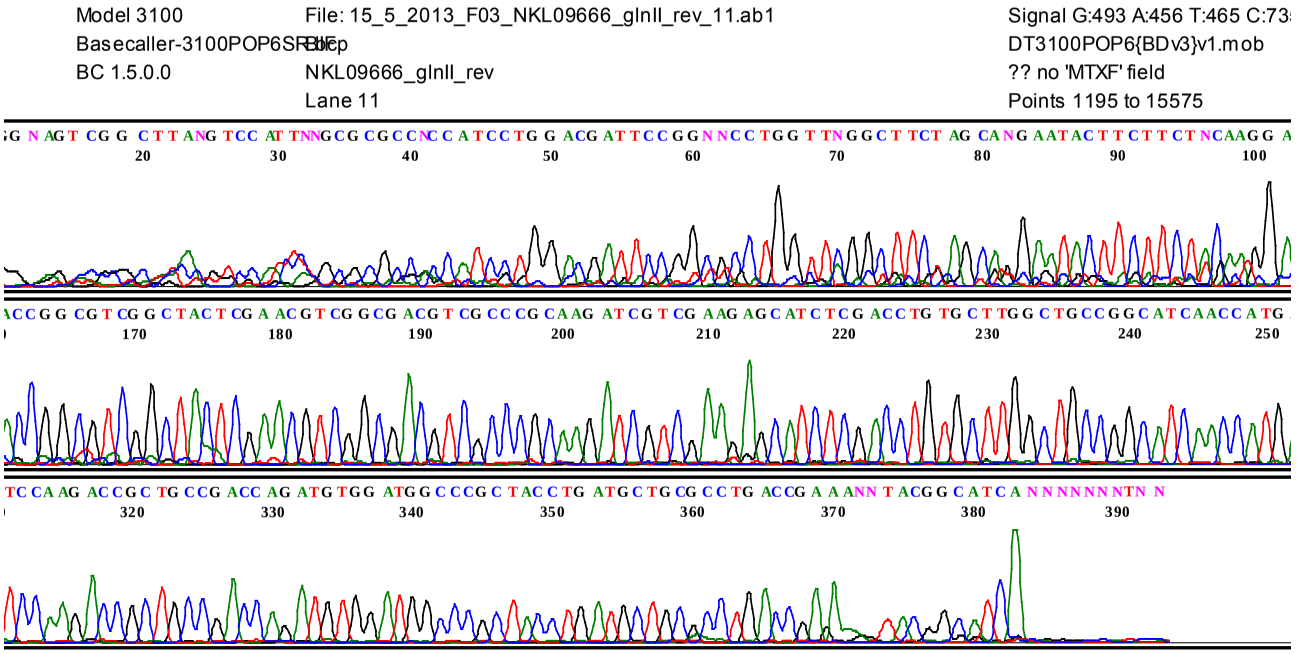

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 15_5_2013_C03_NKL09231_glnII_for_05.ab1
NKL09231_glnII_for
Lane 5
Signal G:625 A:323 T:343 C:44
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1082 to 15575
TNCG AGCC ATCC C ATC TGG TCG GCAG CGG TCTTG G AG CCC TTG C CG AAG ATC TGG AATTCCCAC TGG CCCTTG G CGAC TTC CG CG TTG ATGC
10 20 30 40 50 60 70 80 90
GG CG ACG TCG CCG ACG TTC CG AG AAG CCG ACG CCG G TG TAG TAC GGG G CCC TG CGG CG CGGG AT AG CCGG CG G TCGGG AAG CCG AG CGG ACGG C
160 170 180 190 200 210 220 230 240
AA TGG TGG CGCG CTTG TTGG ACGG ATG CGGGGG TCTTG CCG TCGGG C ATCA TG ACTTC NCAC AT CAC CNATC A NNNNNNNNN
310 320 330 340 350 360 370 380

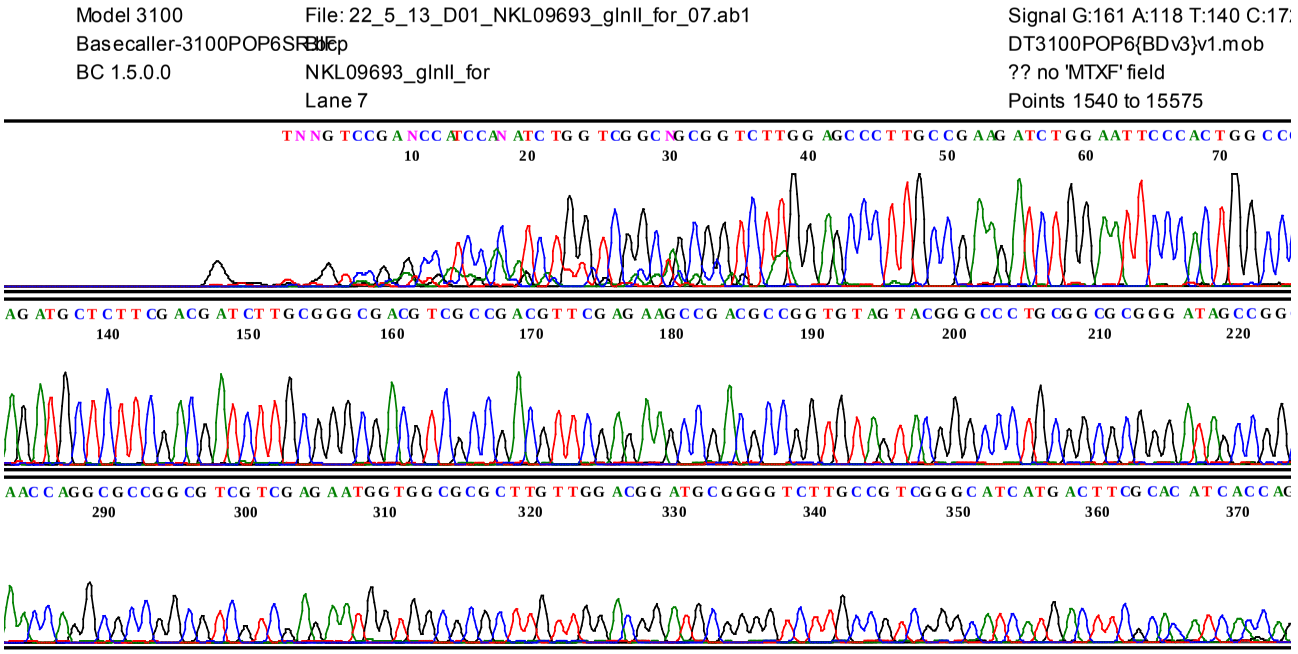

Model 3100
Basecaller-3100POP6SRBE
BC 1.5.0.0
File: 15_5_2013_D03_NKL09231_glnII_rev_07.ab1
Ep
NKL09231_glnII_rev
Lane 7
Signal G:453 A:308 T:318 C:55
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1197 to 15575

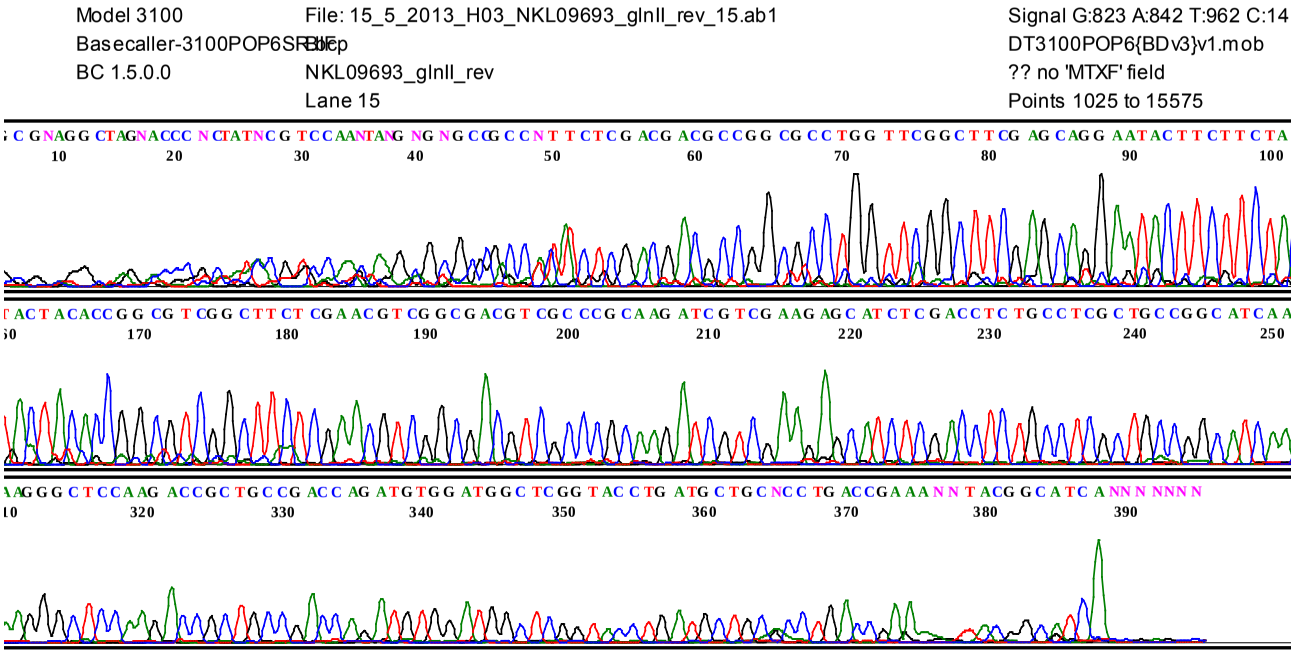

Model 3100
Basecaller-3100POP6SRBDE
BC 1.5.0.0
File: 13_3_13_H01_NKL09273_glhII_f_15.ab1
Bdep
NKL09273_glhII_f
Lane 15
Signal G:171 A:154 T:139 C:21
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 693 to 15575

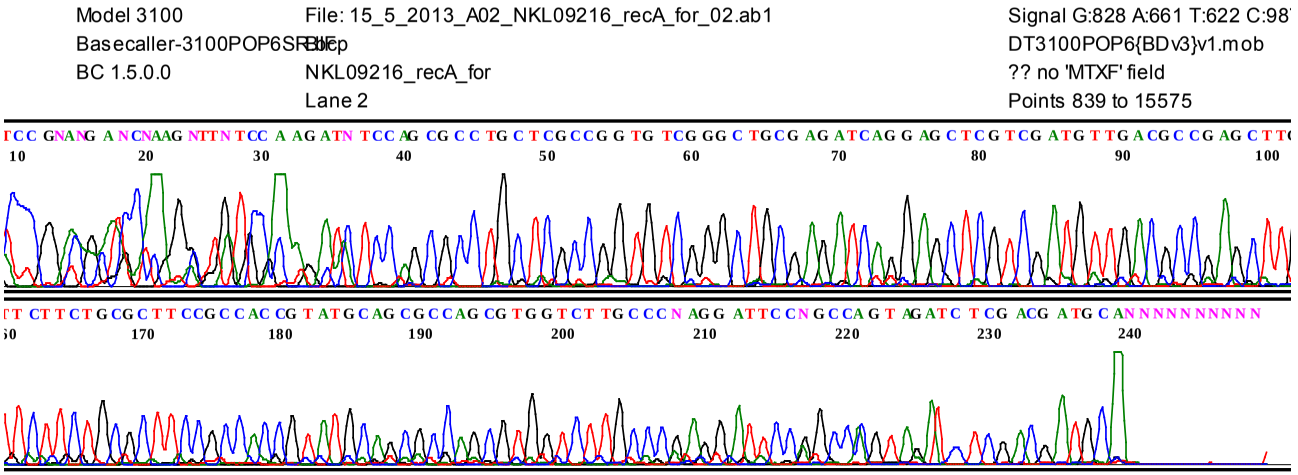

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 13_3_13_A02_NKL09273_glhll_r_02.ab1
NKL09273_glhll_r
Lane 2
Signal G:17 A:14 T:14 C:17
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1088 to 15575

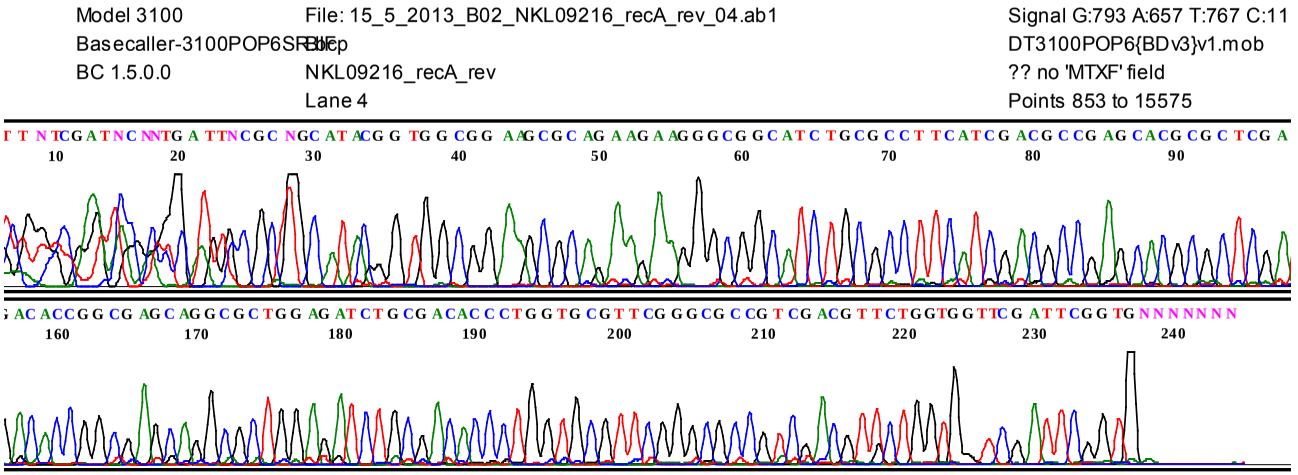

Model 3100
Basecaller-3100POP6SRBDF
BC 1.5.0.0
File: 15_5_2013_E03_NKL09666_glnII_for_09.ab1
Ep
NKL09666_glnII_for
Lane 9
Signal G:657 A:365 T:382 C:50
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1279 to 15575

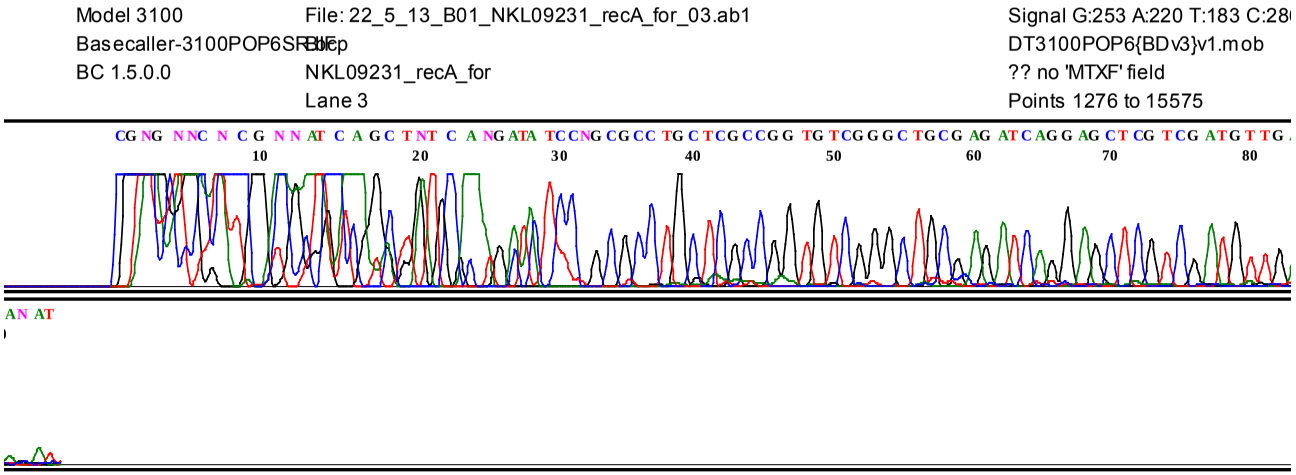

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 15_5_2013_F03_NKL09666_glnII_rev_11.ab1
NKL09666_glnII_rev
Lane 11
Signal G:493 A:456 T:465 C:73
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1195 to 15575

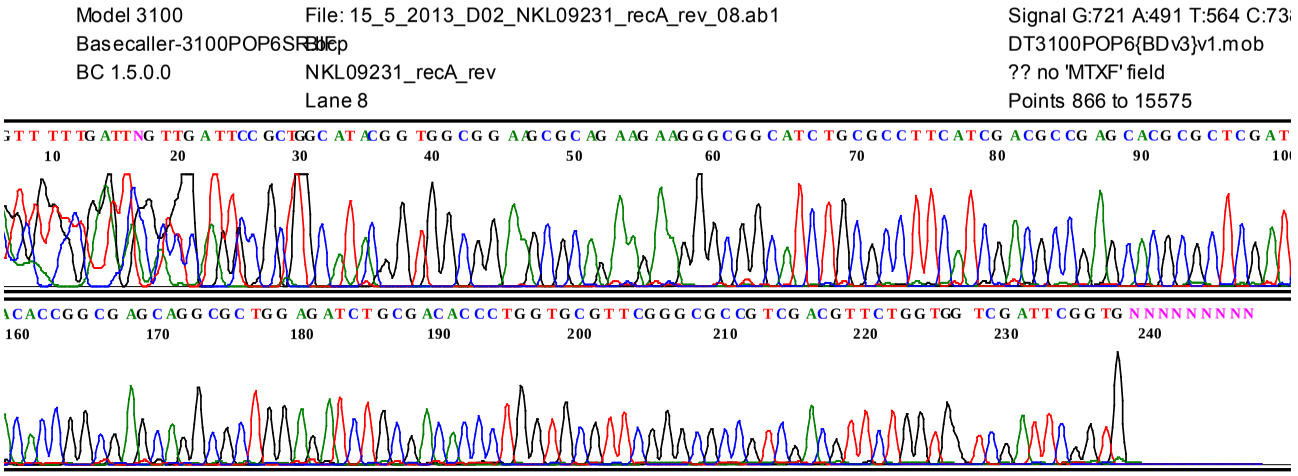

Model 3100
Basecaller-3100POP6SRBDF
BC 1.5.0.0
File: 22_5_13_D01_NKL09693_glnII_for_07.ab1
Bdcp
NKL09693_glnII_for
Lane 7
Signal G:161 A:118 T:140 C:17
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1540 to 15575

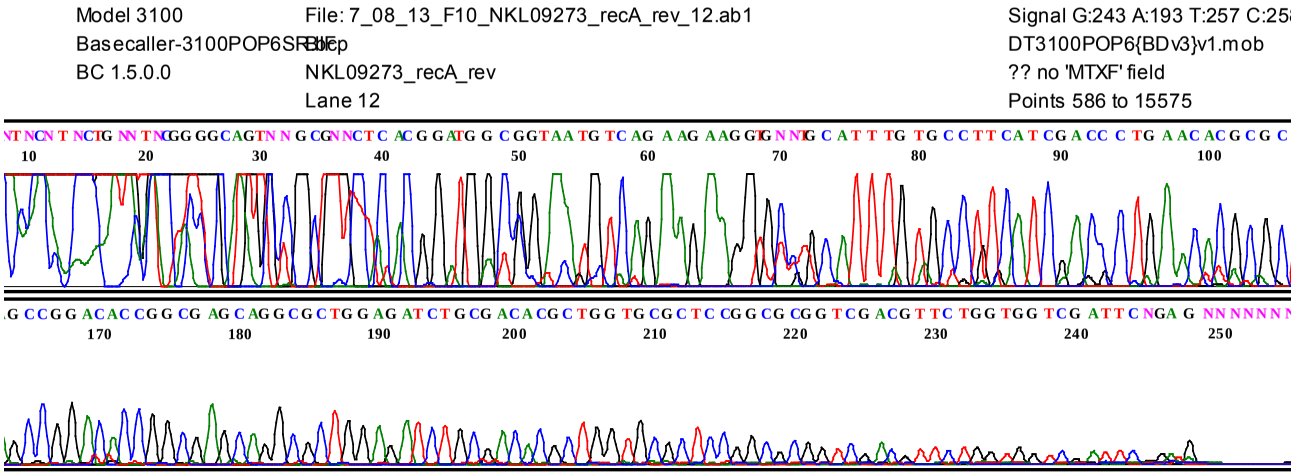

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 15_5_2013_H03_NKL09693_glnII_rev_15.ab1
NKL09693_glnII_rev
Lane 15
Signal G:823 A:842 T:962 C:14
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1025 to 15575

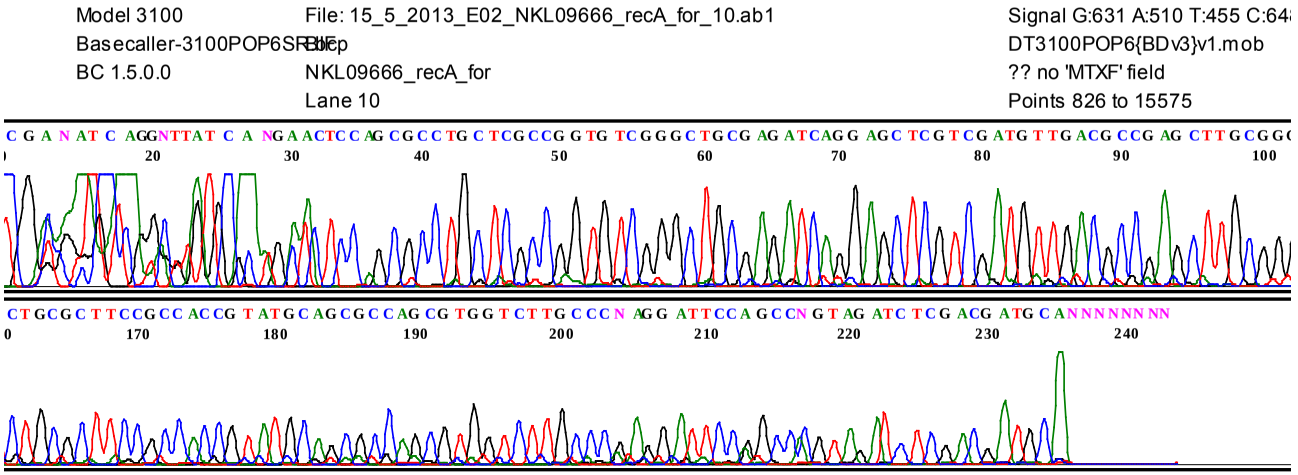

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 15_5_2013_A02_NKL09216_recA_for_02.ab1
NKL09216_recA_for
Lane 2
Signal G:828 A:661 T:622 C:98
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 839 to 15575

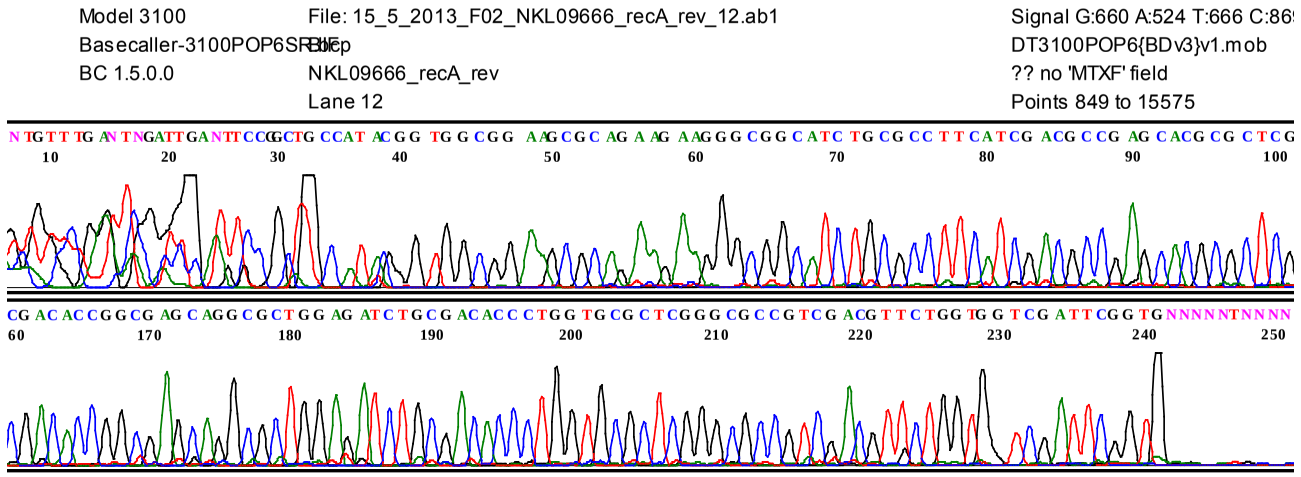

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 15_5_2013_B02_NKL09216_recA_rev_04.ab1
NKL09216_recA_rev
Lane 4
Signal G:793 A:657 T:767 C:11
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 853 to 15575

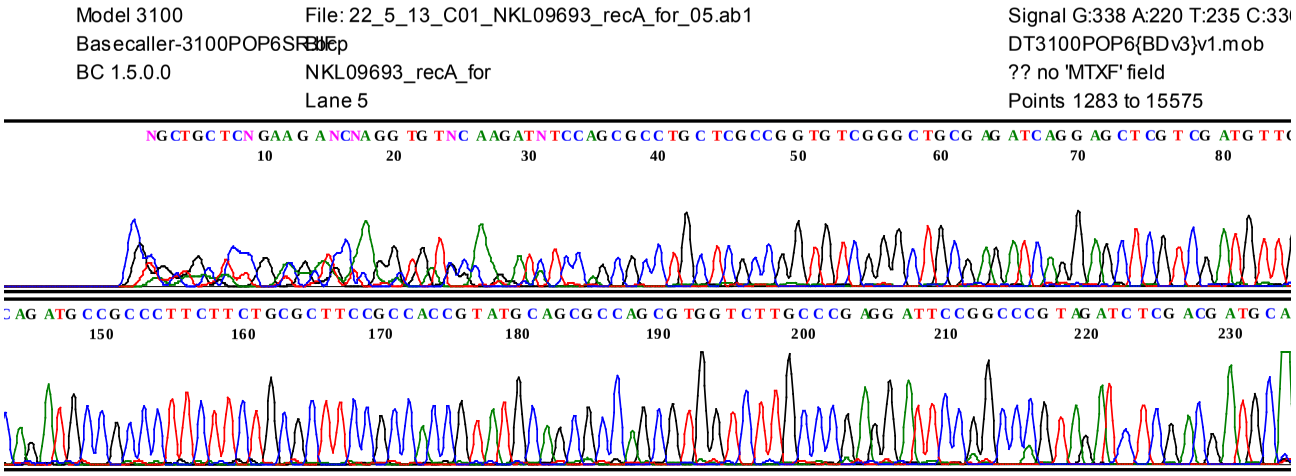

Model 3100
Basecaller-3100POP6SRBC
BC 1.5.0.0
File: 22_5_13_B01_NKL09231_recA_for_03.ab1
NKL09231_recA_for
Lane 3
Signal G:253 A:220 T:183 C:28
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1276 to 15575
10
20
30
40
50
60
70
80

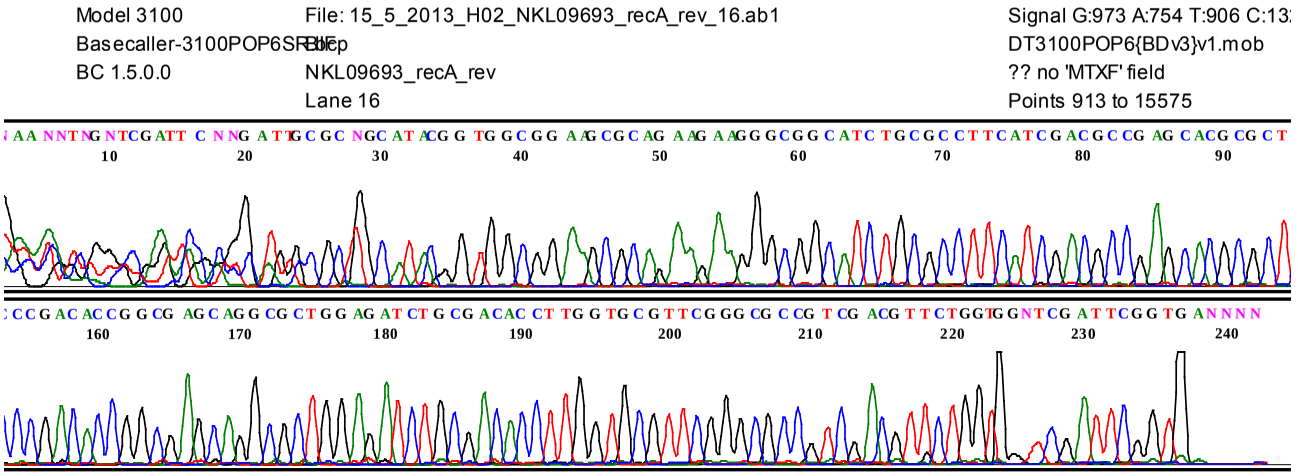

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 15_5_2013_D02_NKL09231_recA_rev_08.ab1
NKL09231_recA_rev
Lane 8
Signal G:721 A:491 T:564 C:73
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 866 to 15575

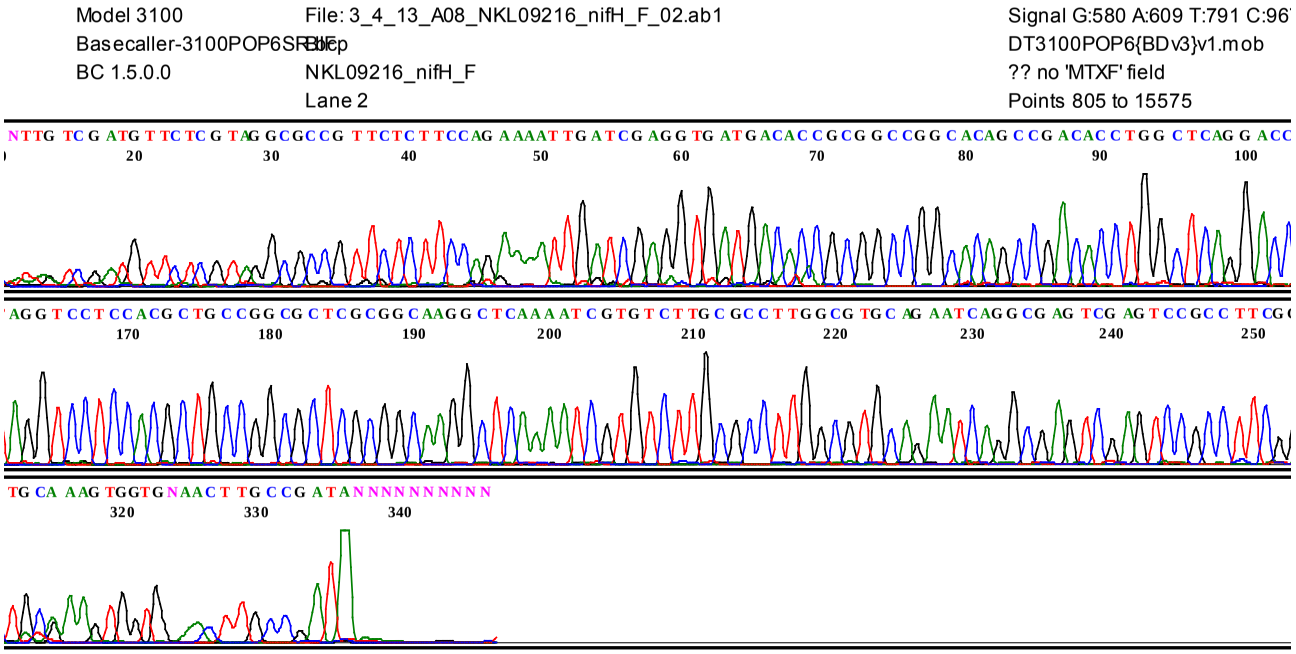

Model 3100
Basecaller-3100POP6SRBDF
BC 1.5.0.0
File: 7_08_13_F10_NKL09273_recA_rev_12.ab1
Ep
NKL09273_recA_rev
Lane 12
Signal G:243 A:193 T:257 C:25
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 586 to 15575

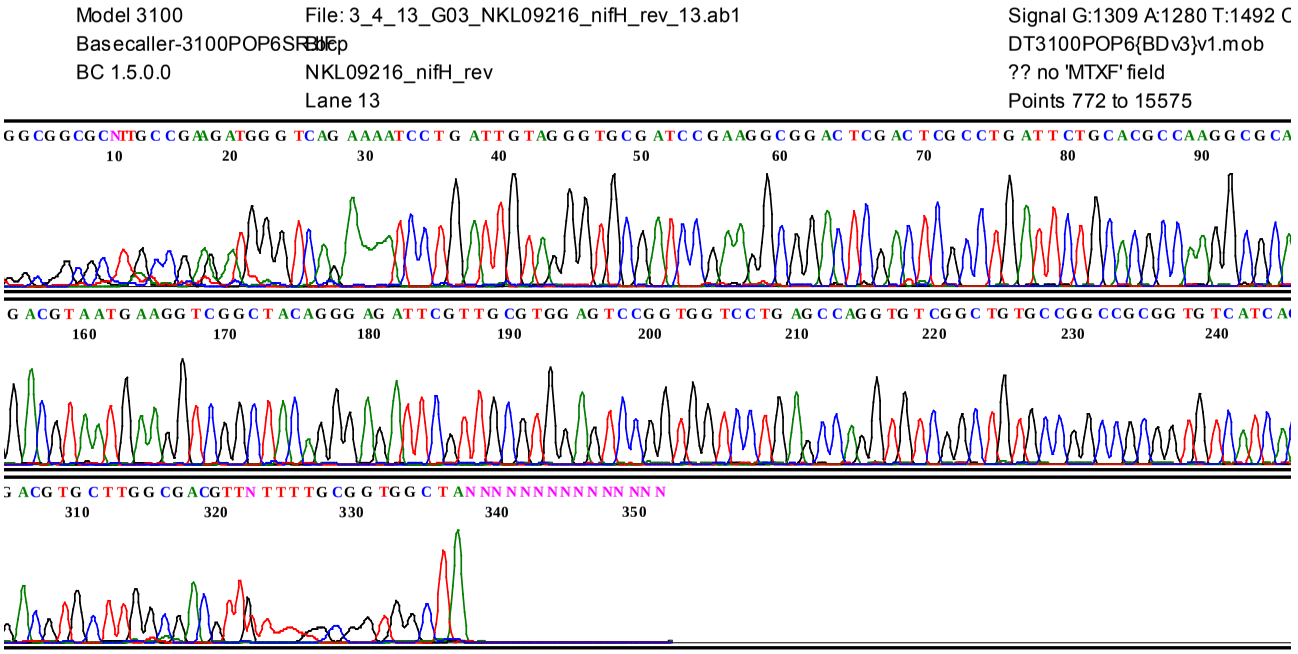

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 15_5_2013_E02_NKL09666_recA_for_10.ab1
NKL09666_recA_for
Lane 10
Signal G:631 A:510 T:455 C:64
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 826 to 15575
C G A N A T C AGGNTTAT C A NGA A CTCC AG C G C C TG C T C G C C G G TG TC G G G C TG C G AG A TC AG G AG C TC G T C G A TG T T G A C G C C G AG C T T G C G G C
20 30 40 50 60 70 80 90 100
CT GC G C T T C C G C C AC C G T A T G C A G C G C C A G C G TG G T C T T G C C C N AG G A T T C C A G C C N G T AG A T C T C G A C G A TG C A N N N N N N N N
0 170 180 190 200 210 220 230 240

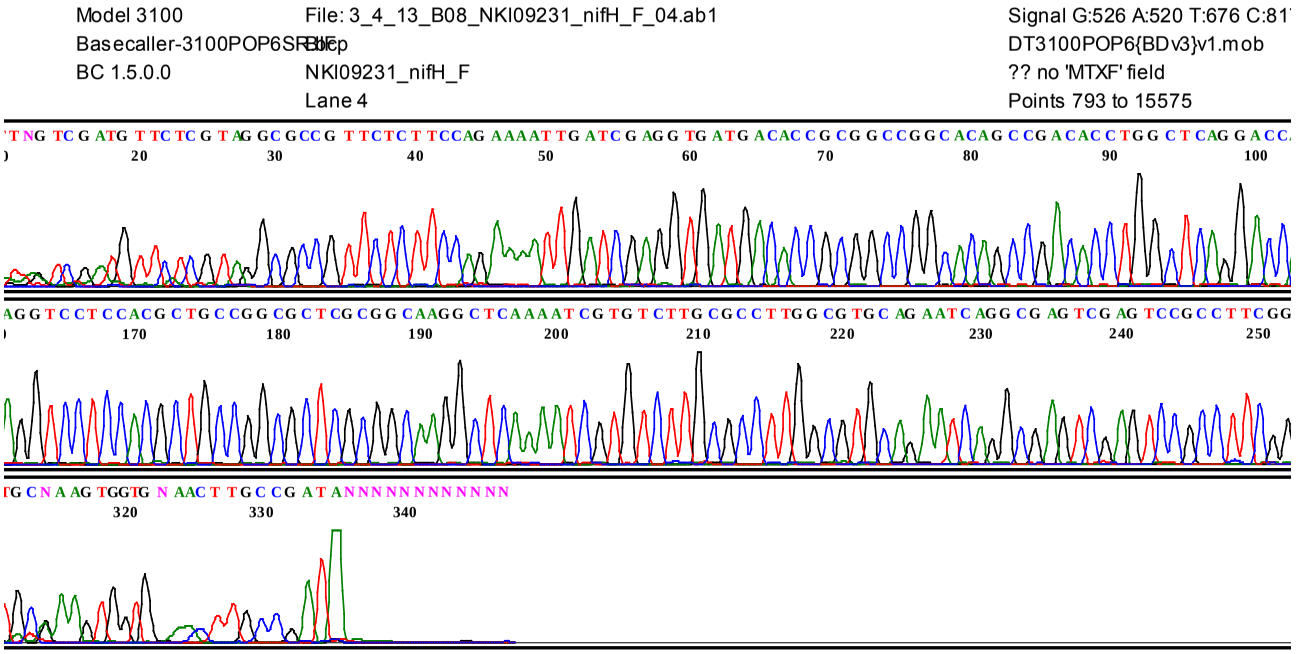

Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 15_5_2013_F02_NKL09666_recA_rev_12.ab1
NKL09666_recA_rev
Lane 12
Signal G:660 A:524 T:666 C:86
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 849 to 15575

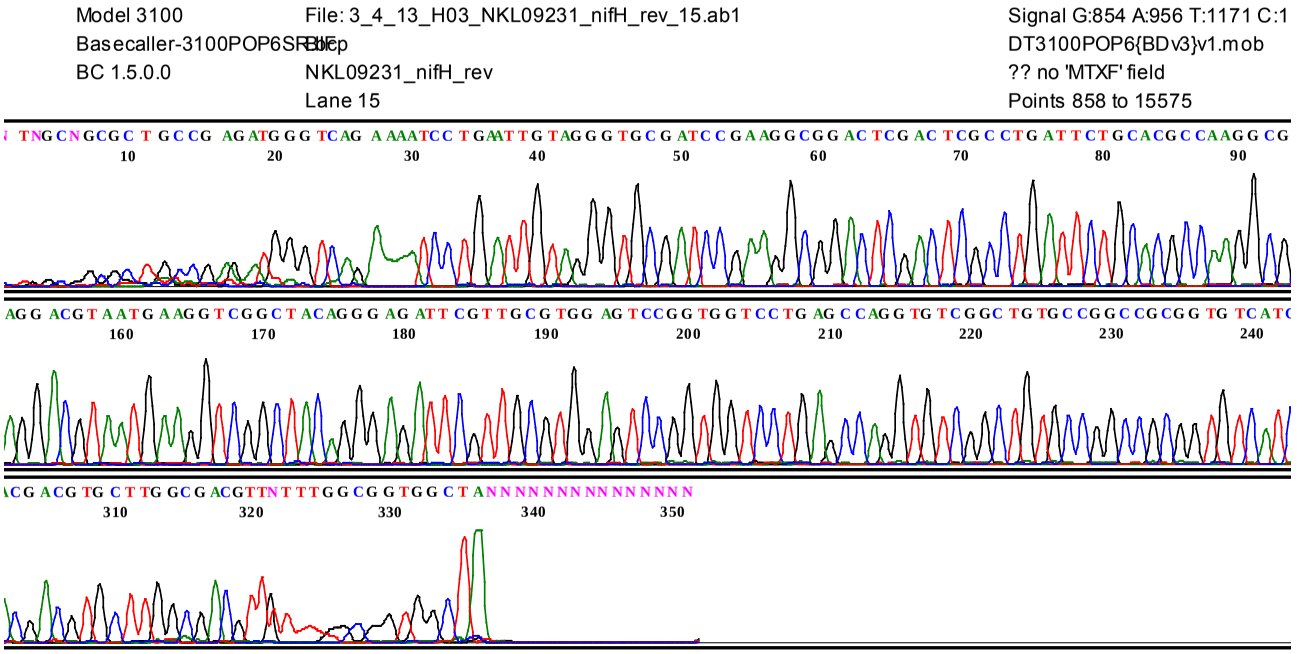

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 22_5_13_C01_NKL09693_recA_for_05.ab1
NKL09693_recA_for
Lane 5
Signal G:338 A:220 T:235 C:33
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1283 to 15575
10
20
30
40
50
60
70
80
150
160
170
180
190
200
210
220
230

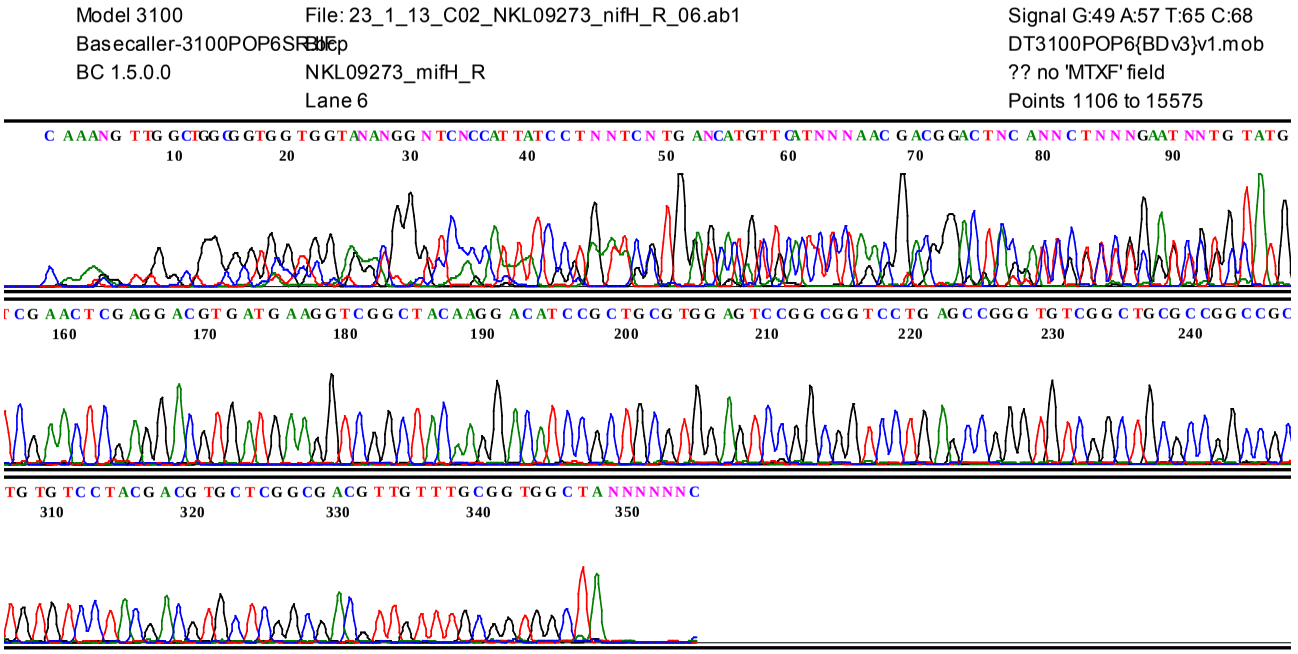

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 15_5_2013_H02_NKL09693_recA_rev_16.ab1
NKL09693_recA_rev
Lane 16
Signal G:973 A:754 T:906 C:13
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 913 to 15575

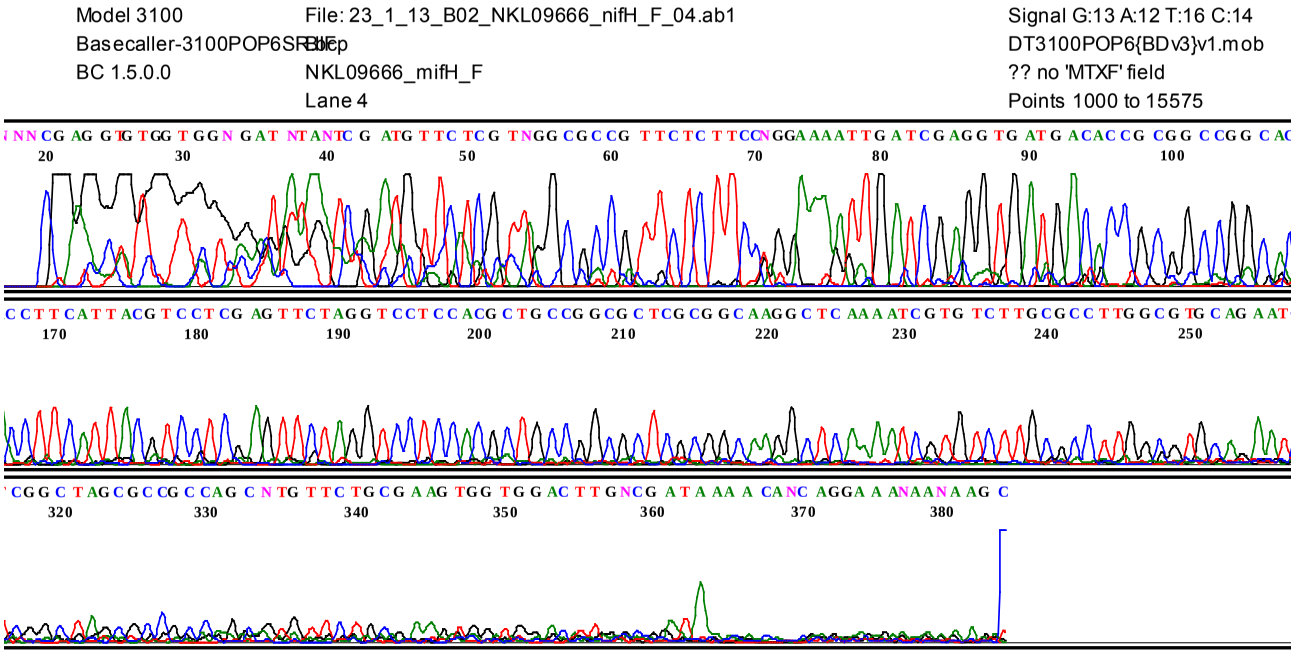

Model 3100
Basecaller-3100POP6SRBDF
BC 1.5.0.0
File: 3_4_13_A08_NKL09216_nifH_F_02.ab1
Bdcp
NKL09216_nifH_F
Lane 2
Signal G:580 A:609 T:791 C:96
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 805 to 15575
NTTG TC G ATG T TC TC G TAG GCGCCG T TCTC T T CCAG AAAAT T G A T C G AG G TG ATG A CAC C G C G G C C G G C ACAG C C G ACAC C T G G C T CAG G ACC
20 30 40 50 60 70 80 90 100
AG G T C C T CC ACG C T G C C G G C G C T C G C G G C AAG G C T C AAA A AT C G T G T C T T G C G C C T T G G C G TG C AG AAT C AG G C G AG T C G AG T C C G C C T T C G
170 180 190 200 210 220 230 240 250
TG CA AAG TGGTG NAAC T T G C C G A T A NNNNNNNNNN
320 330 340

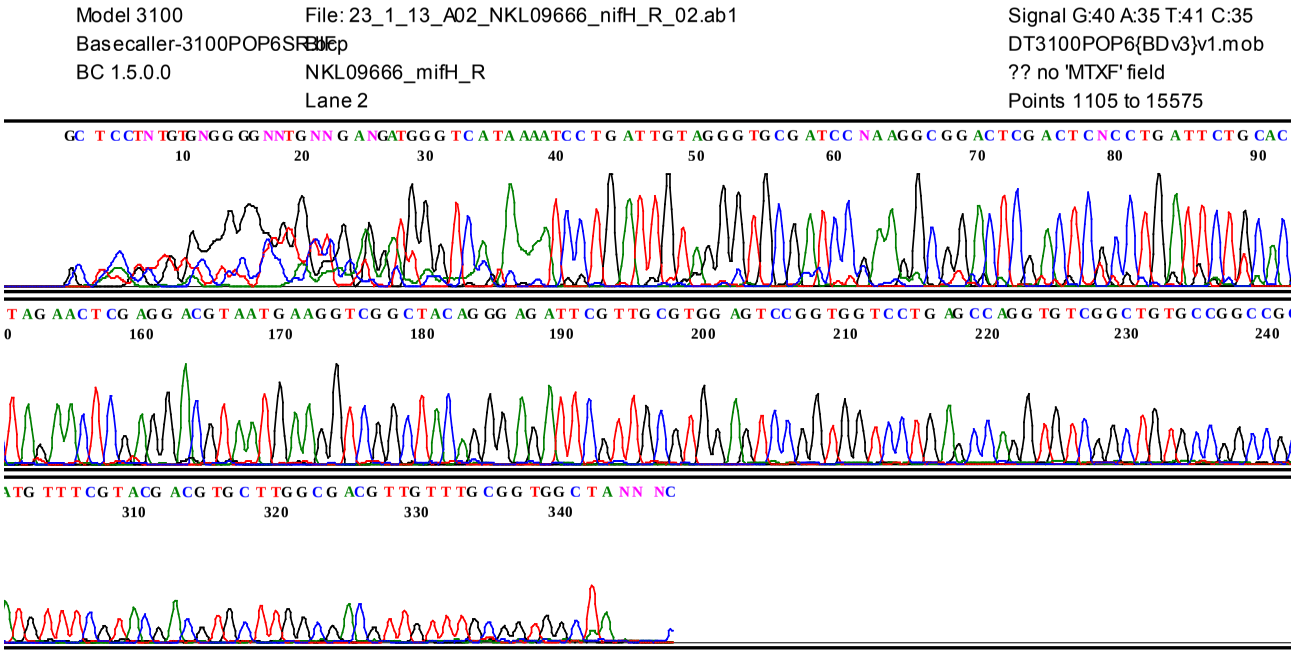

Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 3_4_13_G03_NKL09216_nifH_rev_13.ab1
NKL09216_nifH_rev
Lane 13
Signal G:1309 A:1280 T:1492 C
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 772 to 15575

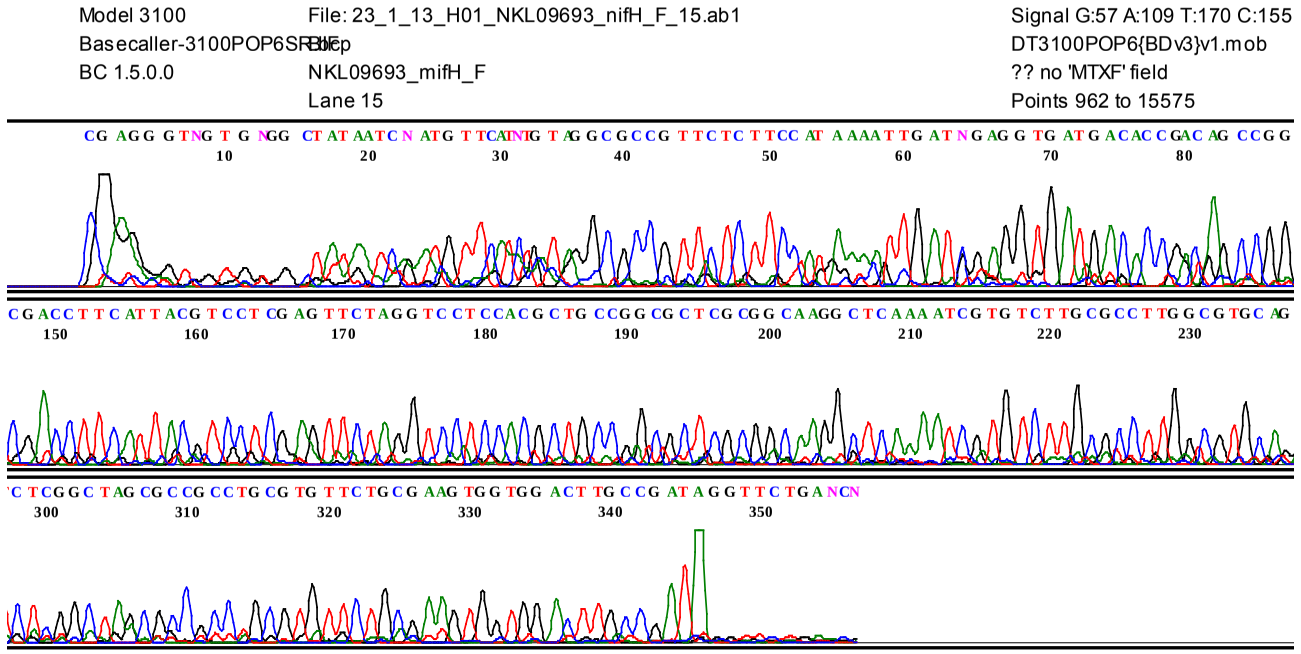

Model 3100
Basecaller-3100POP6SRBDF
BC 1.5.0.0
File: 3_4_13_B08_NKI09231_nifH_F_04.ab1
Ep
NKI09231_nifH_F
Lane 4
Signal G:526 A:520 T:676 C:81
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 793 to 15575

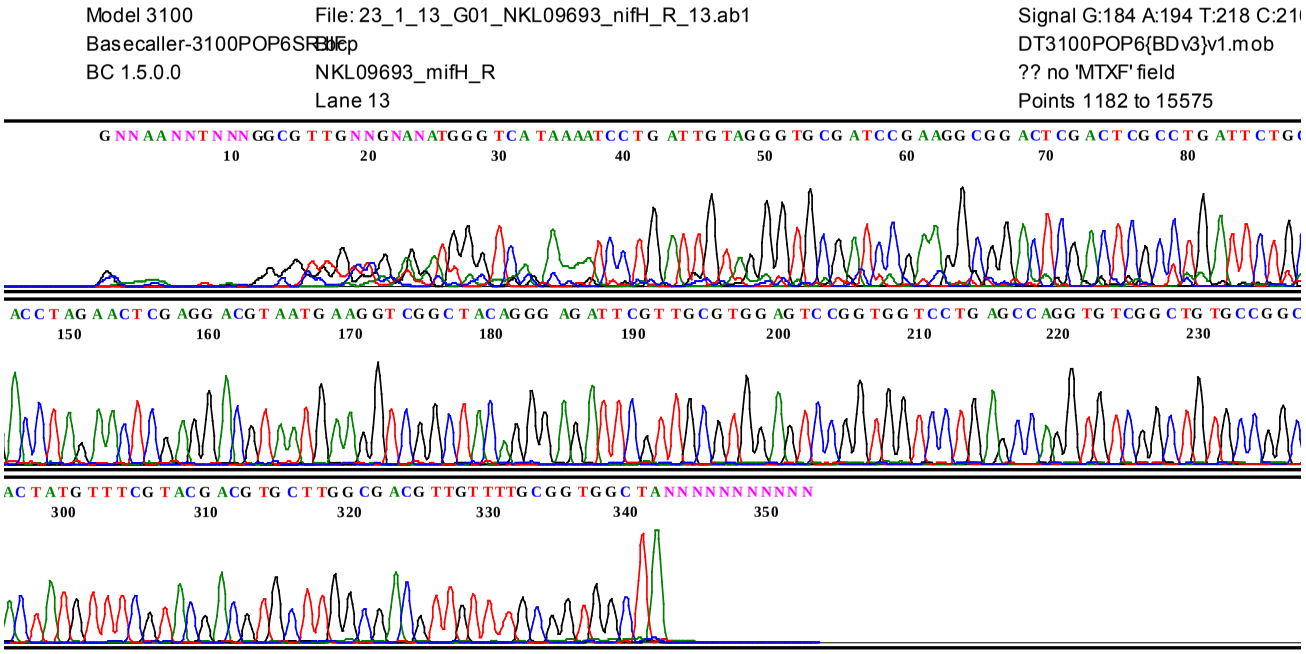

Model 3100
Basecaller-3100POP6SRBC
BC 1.5.0.0
File: 3_4_13_H03_NKL09231_nifH_rev_15.ab1
NKL09231_nifH_rev
Lane 15
Signal G:854 A:956 T:1171 C:1
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 858 to 15575


Model 3100
Basecaller-3100POP6SRBEp
BC 1.5.0.0
File: 23_1_13_C02_NKL09273_nifH_R_06.ab1
NKL09273_mifH_R
Lane 6
Signal G:49 A:57 T:65 C:68
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1106 to 15575


Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 23_1_13_B02_NKL09666_nifH_F_04.ab1
NKL09666_mifH_F
Lane 4
Signal G:13 A:12 T:16 C:14
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1000 to 15575


Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 23_1_13_A02_NKL09666_nifH_R_02.ab1
NKL09666_mifH_R
Lane 2
Signal G:40 A:35 T:41 C:35
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1105 to 15575


Model 3100
Basecaller-3100POP6SRBDfp
BC 1.5.0.0
File: 23_1_13_H01_NKL09693_nifH_F_15.ab1
NKL09693_mifH_F
Lane 15
Signal G:57 A:109 T:170 C:155
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 962 to 15575


Model 3100
Basecaller-3100POP6SRBDEp
BC 1.5.0.0
File: 23_1_13_G01_NKL09693_nifH_R_13.ab1
NKL09693_mifH_R
Lane 13
Signal G:184 A:194 T:218 C:21
DT3100POP6{BDv3}v1.mob
?? no 'MTXF' field
Points 1182 to 15575

APPENDIX I

SEQUENCES OF 16S rDNA, *dnaK*, *glnII, recA* AND *nifH* GENES

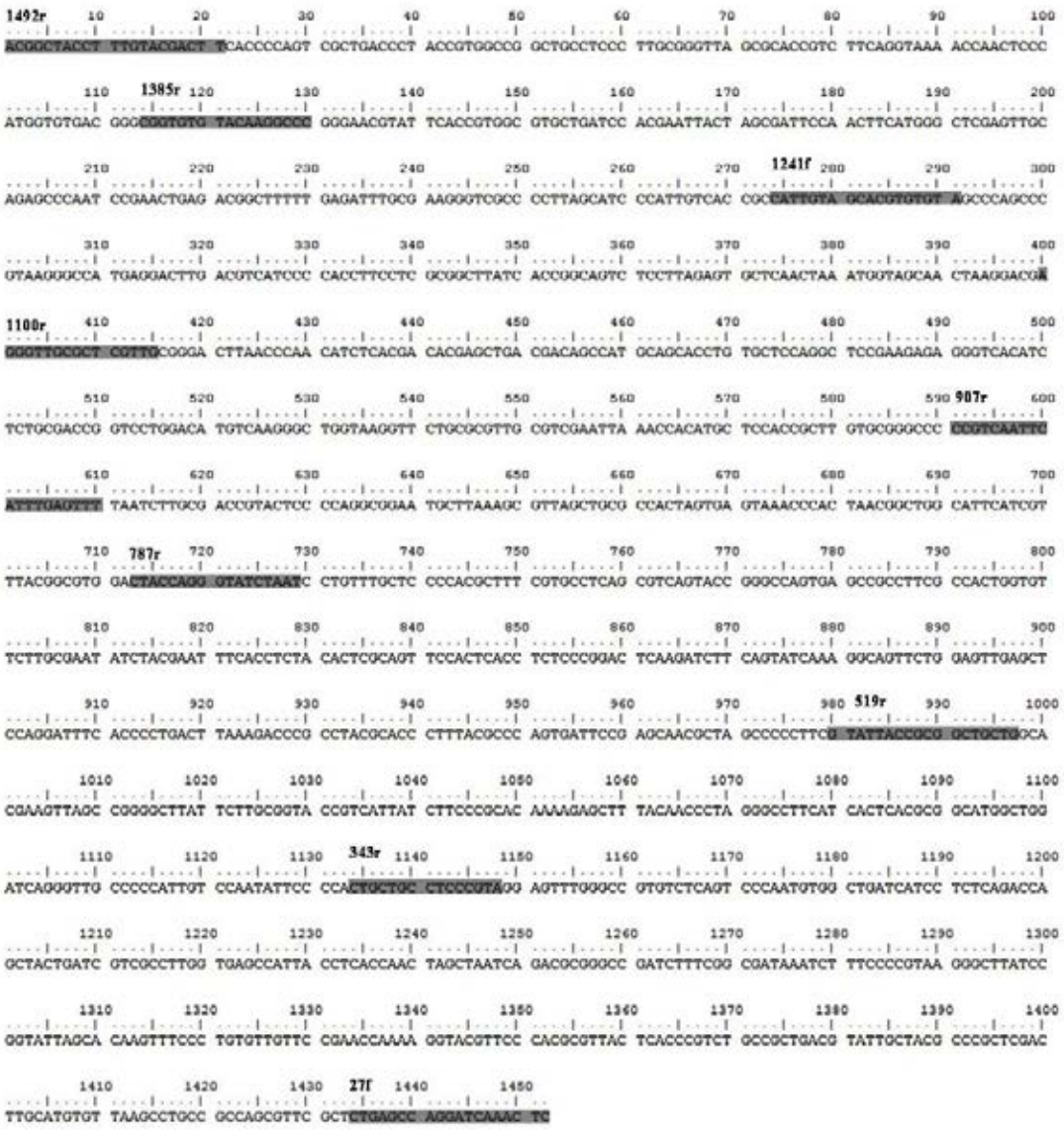


Figure I.1 16S rDNA sequence of NKL09216 with sequences of primers in boxes.

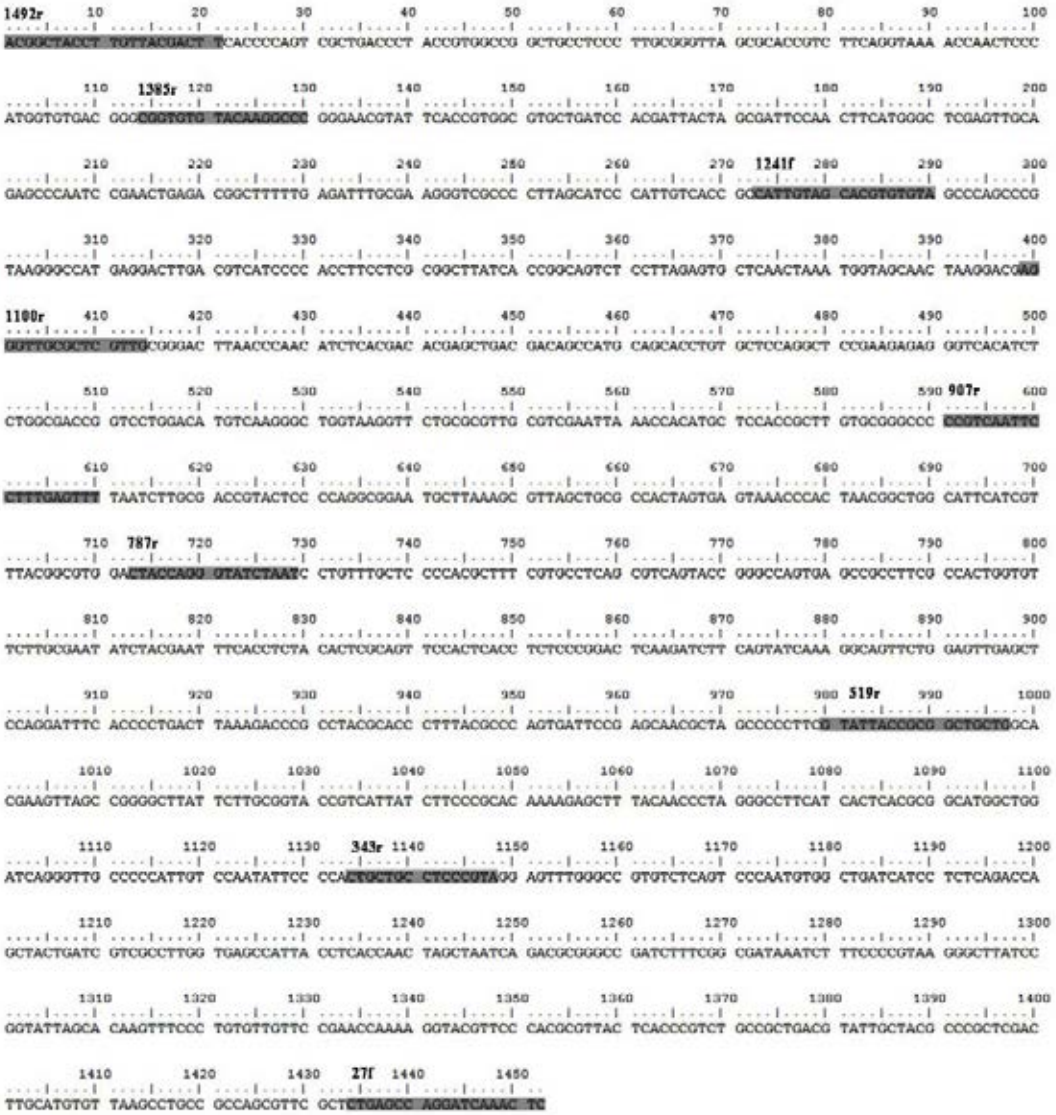


Figure I.2 16S rDNA sequence of NKL09231 with sequences of primers in boxes.

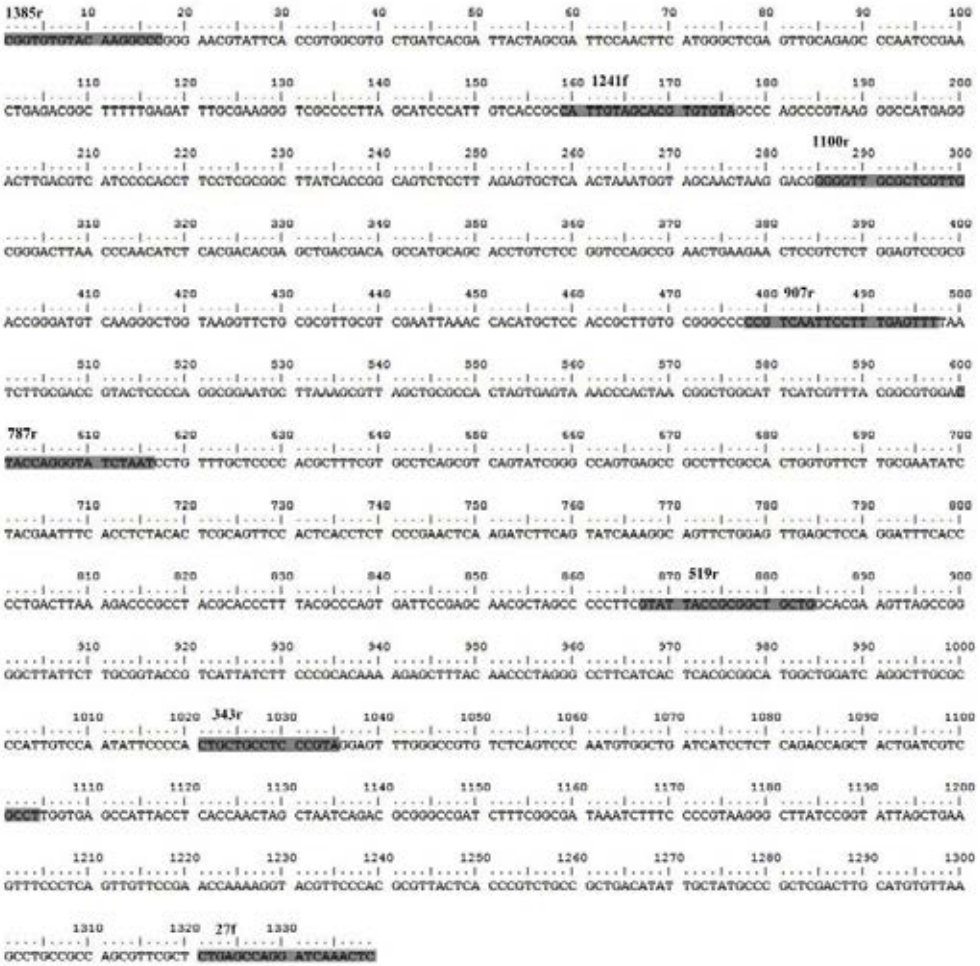


Figure I.3 16S rDNA sequence of NKL09273 with sequences of primers in boxes.

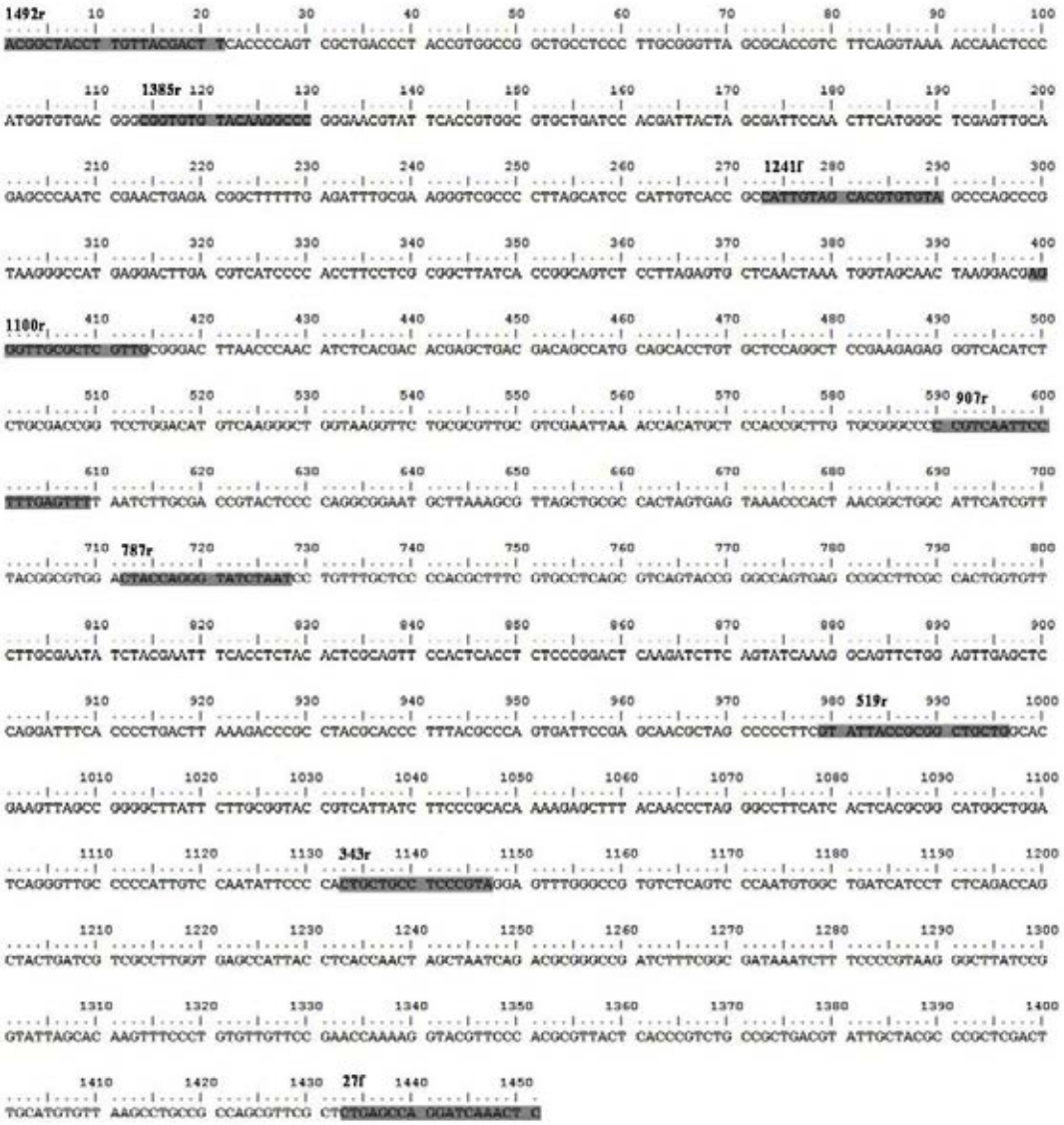


Figure I.4 16S rDNA sequence of NKL09666 with sequences of primers in boxes.

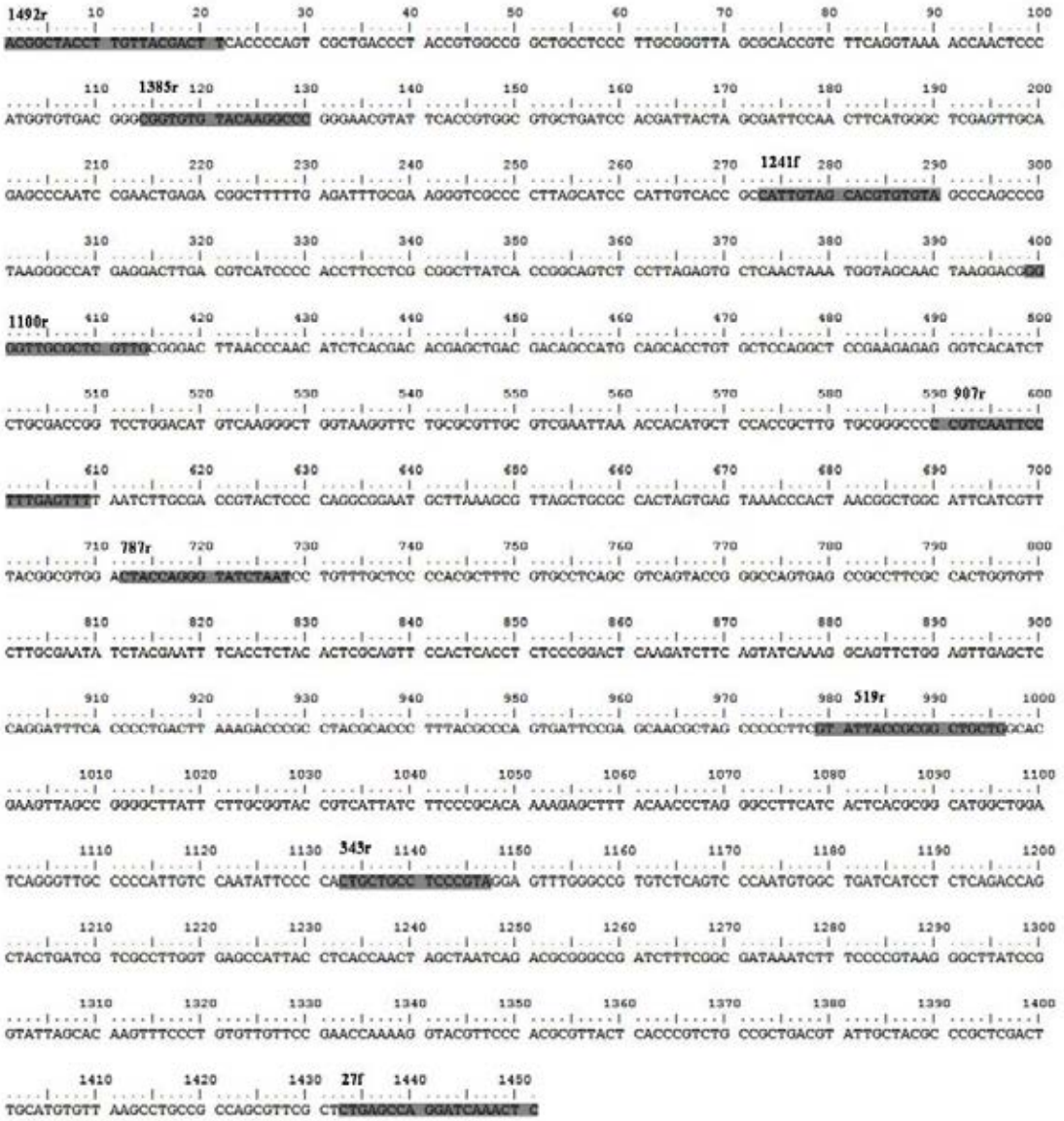


Figure I.5 16S rDNA sequence of NKL09693 with sequences of primers in boxes.

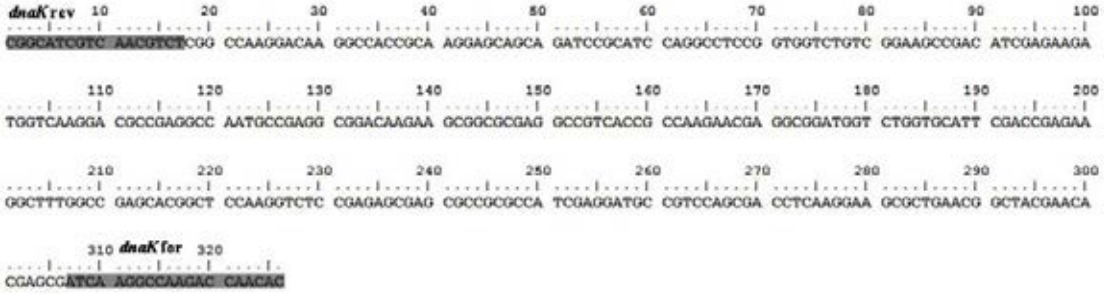


Figure I.6 *dnaK* sequence of NKL09216 with sequences of primers in boxes.

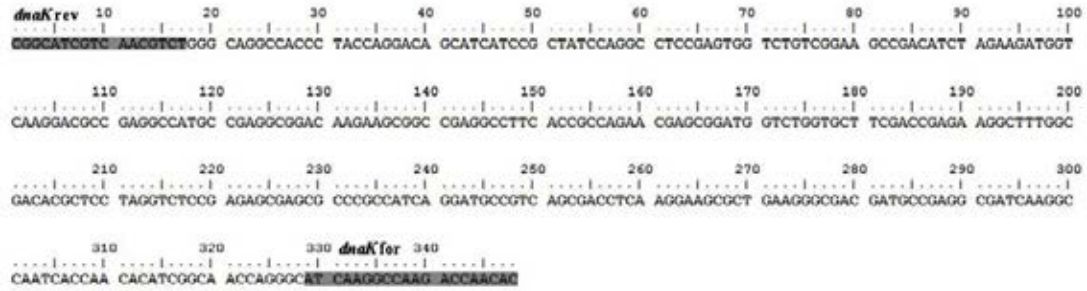


Figure I.7 *dnaK* sequence of NKL09231 with sequences of primers in boxes.

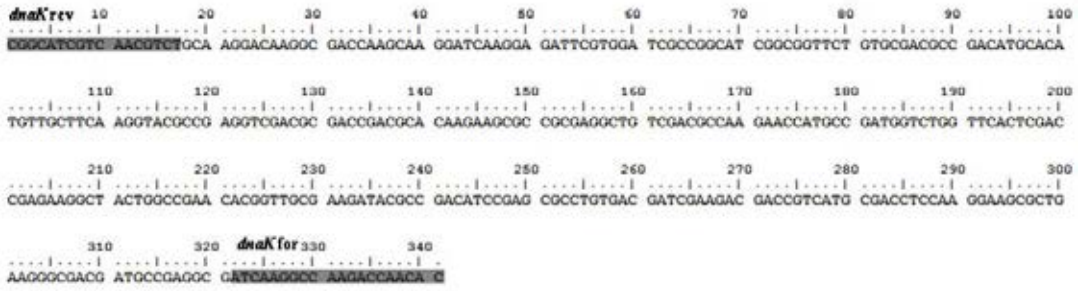


Figure I.8 *dnaK* sequence of NKL09273 with sequences of primers in boxes.

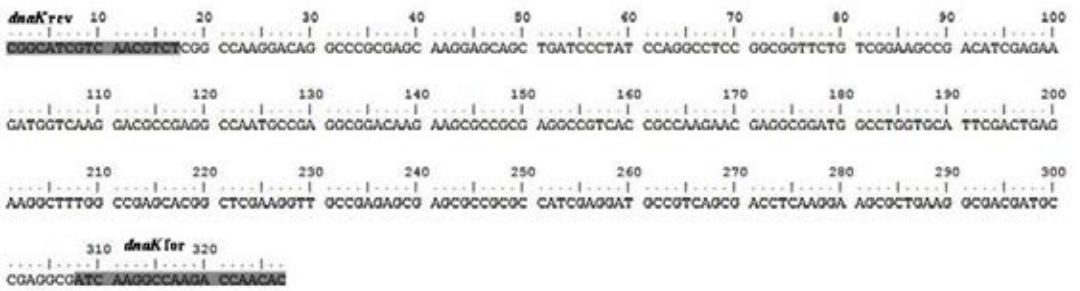


Figure I.9 *dnaK* sequence of NKL09666 with sequences of primers in boxes.

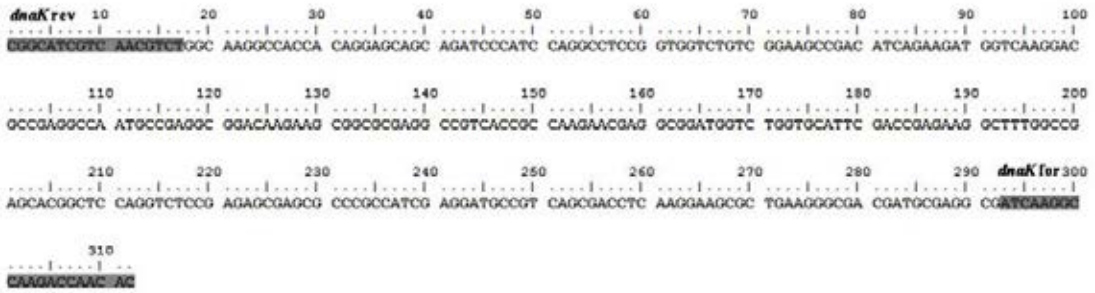


Figure I.10 *dnaK* sequence of NKL09693 with sequences of primers in boxes.

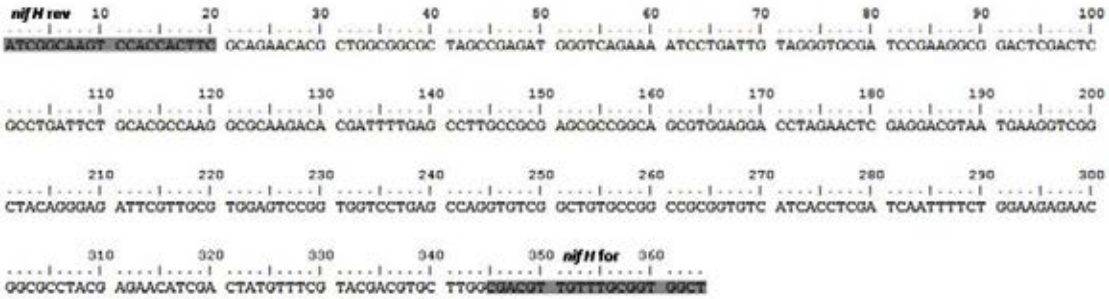


Figure I.11 *nifH* sequence of NKL09216 with sequences of primers in boxes.

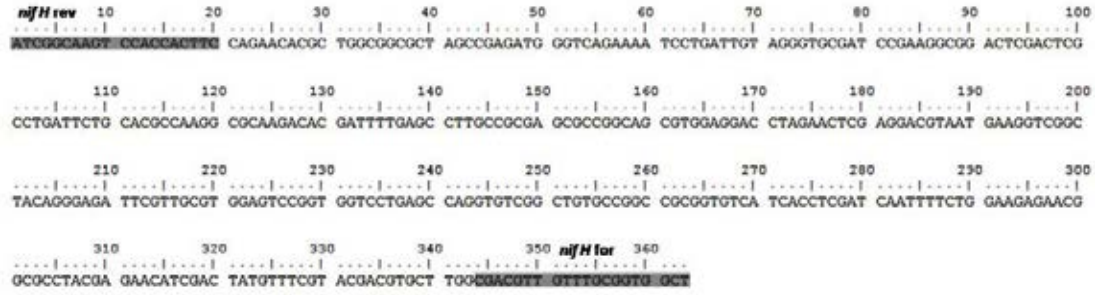


Figure I.12 *nifH* sequence of NKL09231 with sequences of primers in boxes.

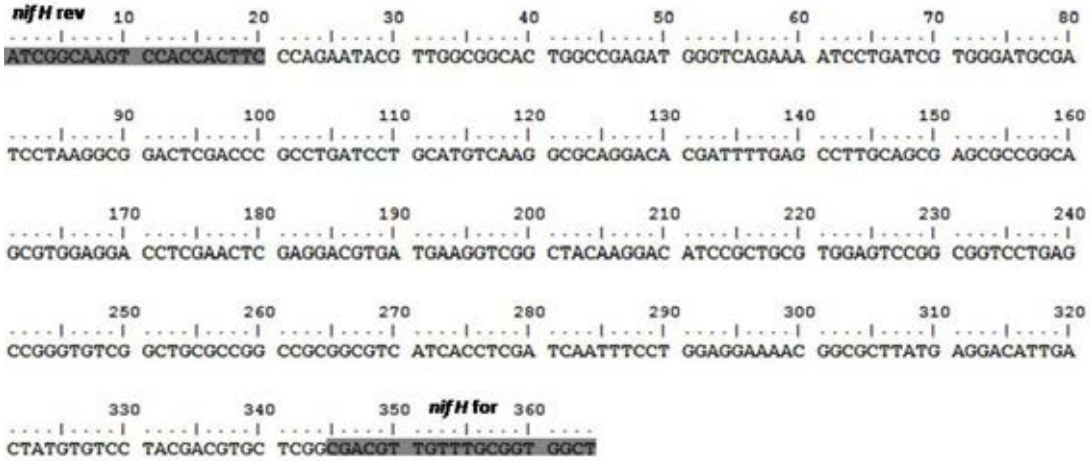


Figure I.13 *nifH* sequence of NKL09273 with sequences of primers in boxes.

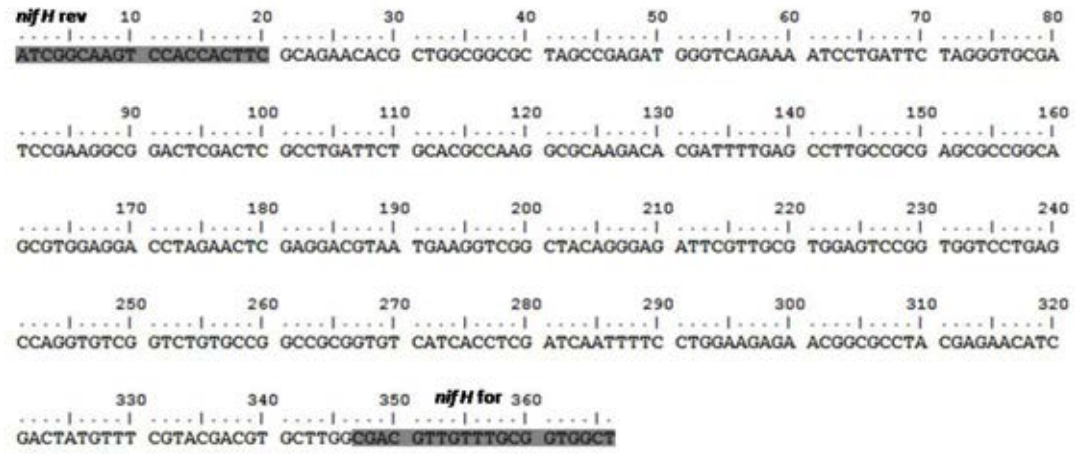


Figure I.14 *nifH* sequence of NKL09666 with sequences of primers in boxes.

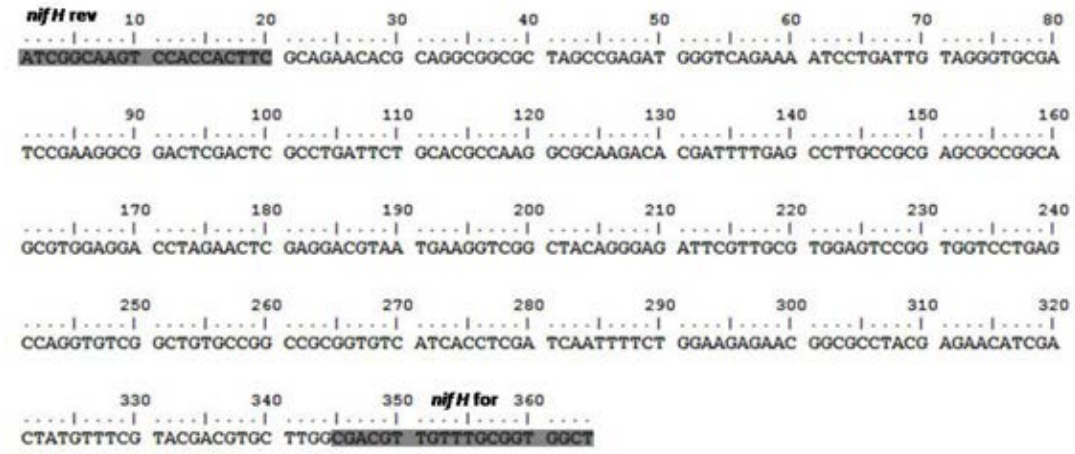


Figure I.15 *nifH* sequence of NKL09693 with sequences of primers in boxes.

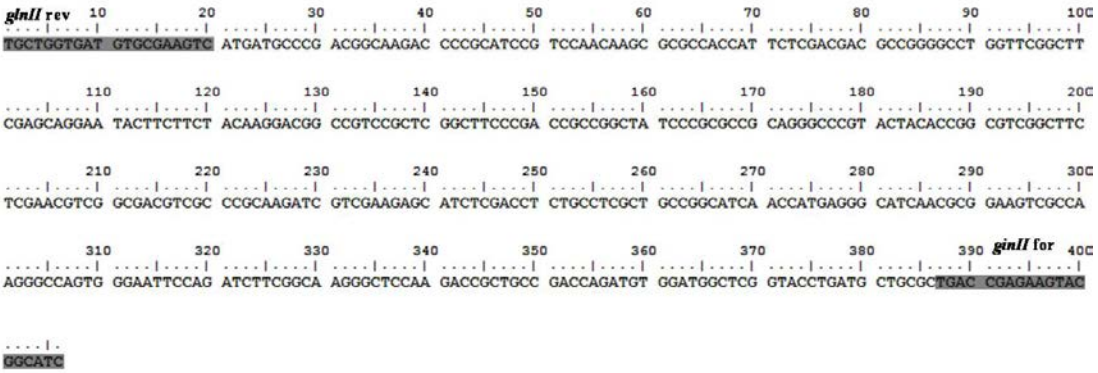


Figure I.16 *glnII* sequence of NKL09216 with sequences of primers in boxes.

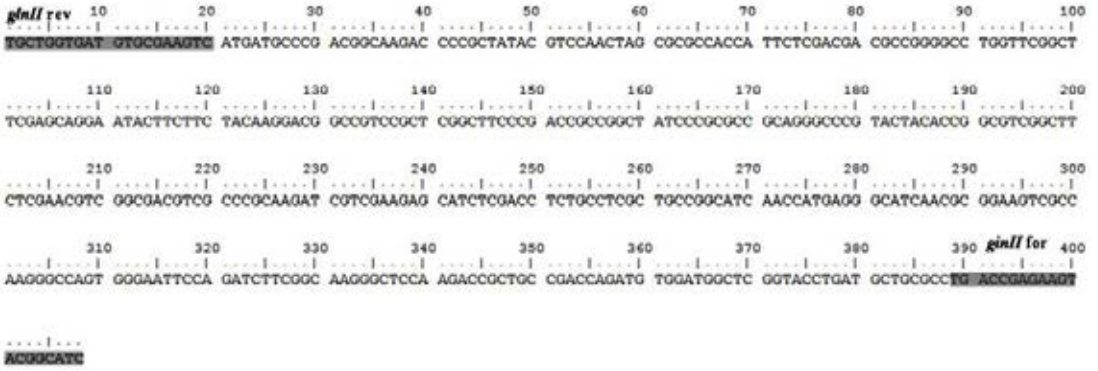


Figure I.17 *glnII* sequence of NKL09231 with sequences of primers in boxes.

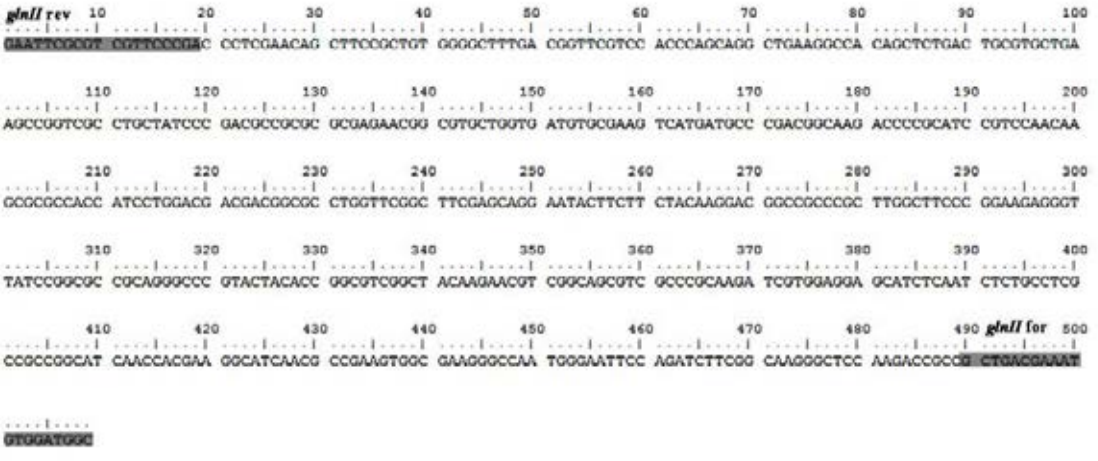


Figure I.18 *glnII* sequence of NKL09273 with sequences of primers in boxes.

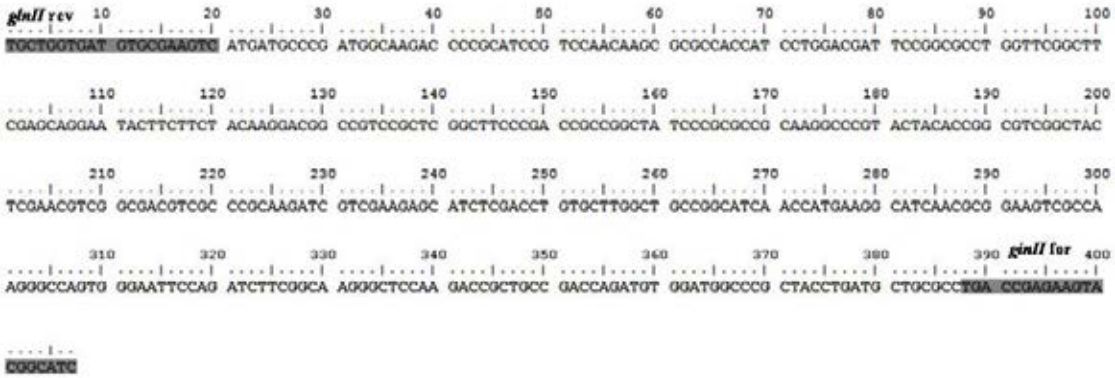


Figure I.19 *glnII* sequence of NKL09666 with sequences of primers in boxes.

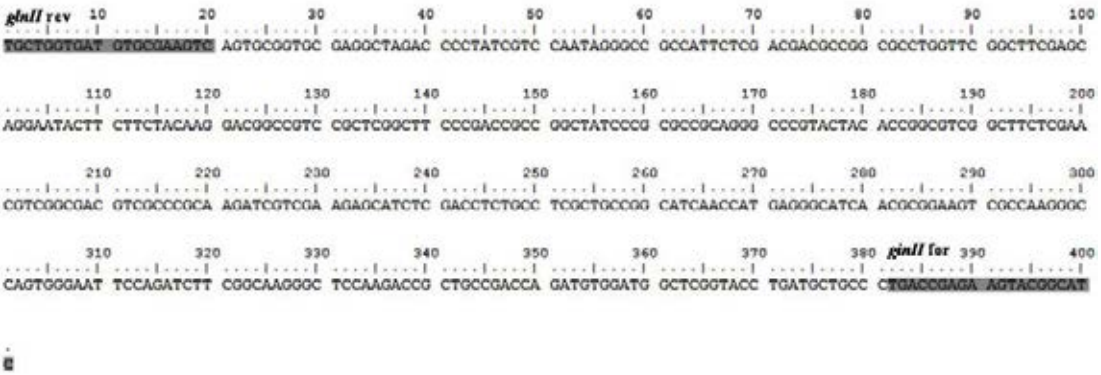


Figure I.20 *glnII* sequence of NKL09693 with sequences of primers in boxes.

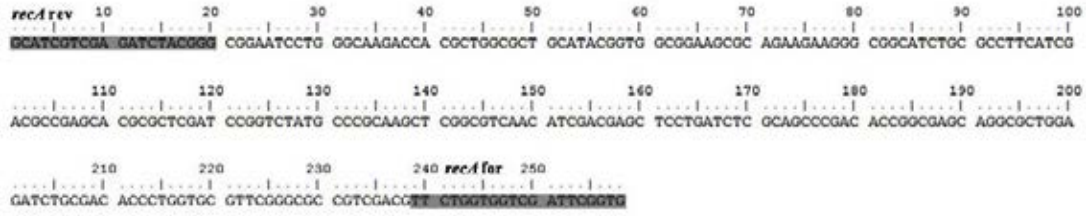


Figure I.21 *recA* sequence of NKL09216 with sequences of primers in boxes.

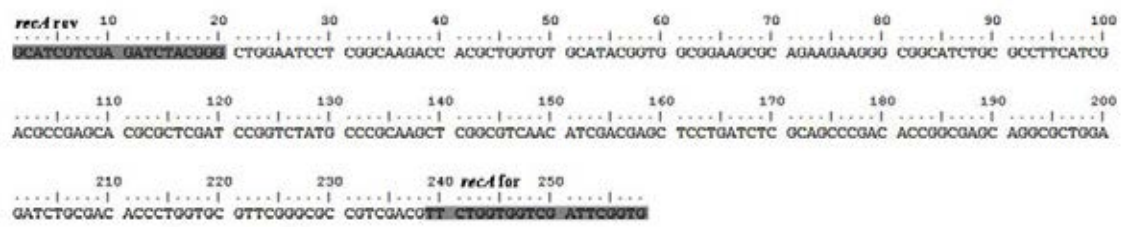


Figure I.22 *recA* sequence of NKL09231 with sequences of primers in boxes.

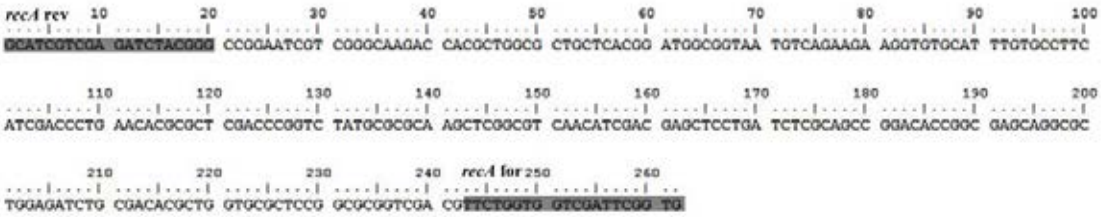


Figure I.23 *recA* sequence of NKL09273 with sequences of primers in boxes.

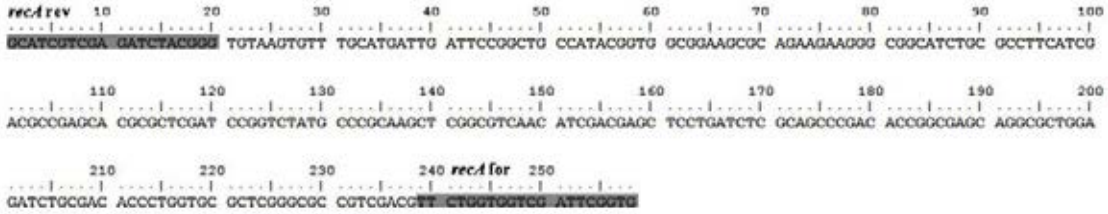


Figure I.24 *recA* sequence of NKL09666 with sequences of primers in boxes.

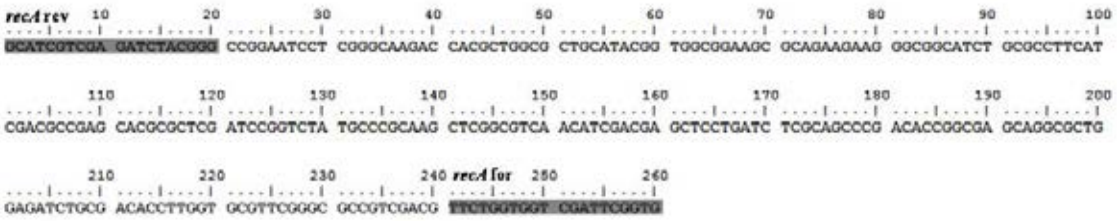


Figure I.25 *recA* sequence of NKL09273 with sequences of primers in boxes.

APPENDIX J

PHYLOGENETIC TREES OF 16S rDNA, *dnaK*, *glnII, recA* AND *nifH* GENES BY MAXIMUM LIKELIHOOD AND NEIGHBOR-JOINING METHOD

MAXIMUM LIKELIHOOD METHOD

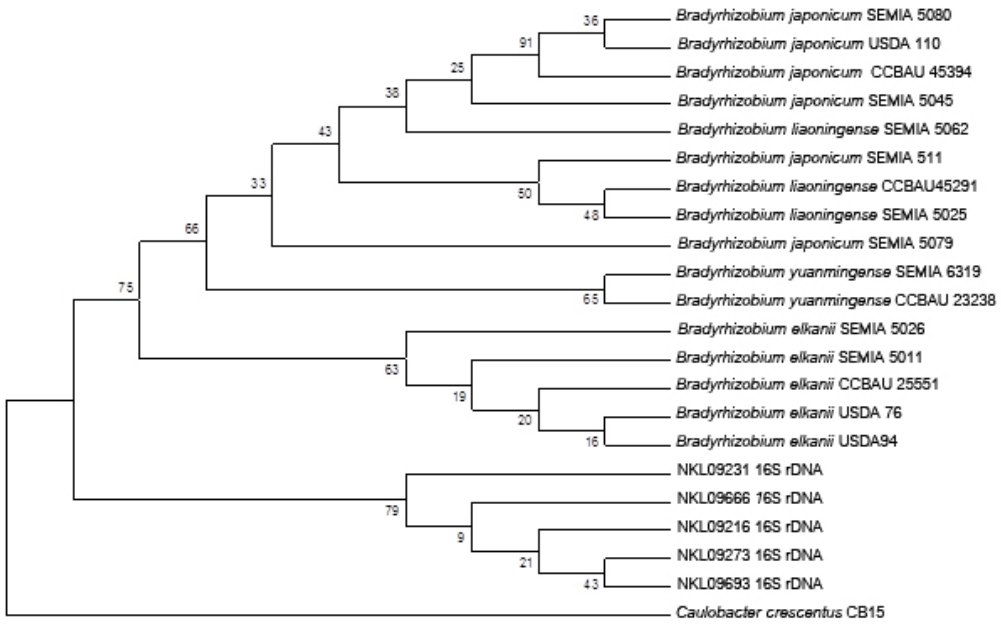


Figure J.1 ML dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of 16S rDNA.

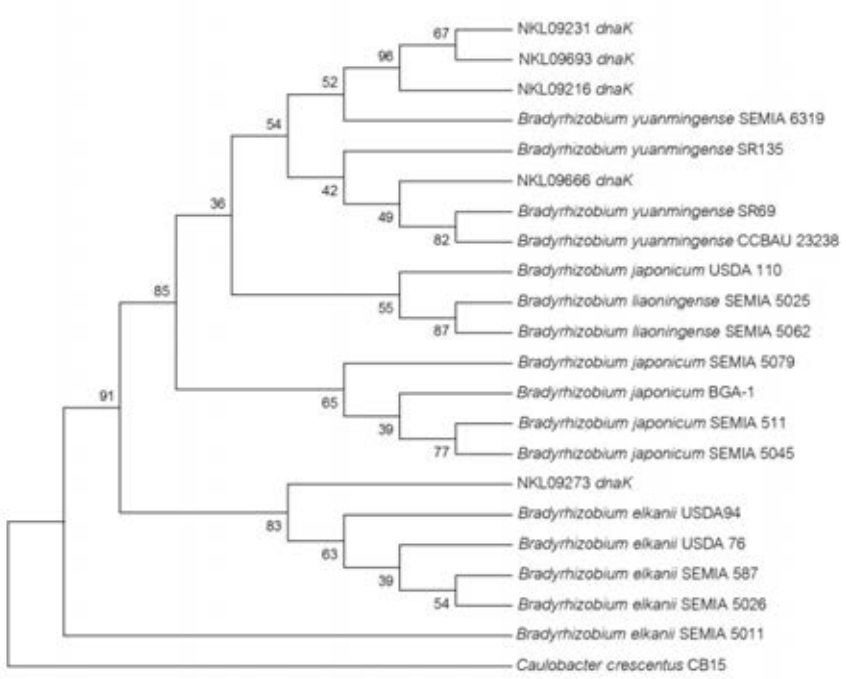


Figure J.2 ML dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of *dnaK.*

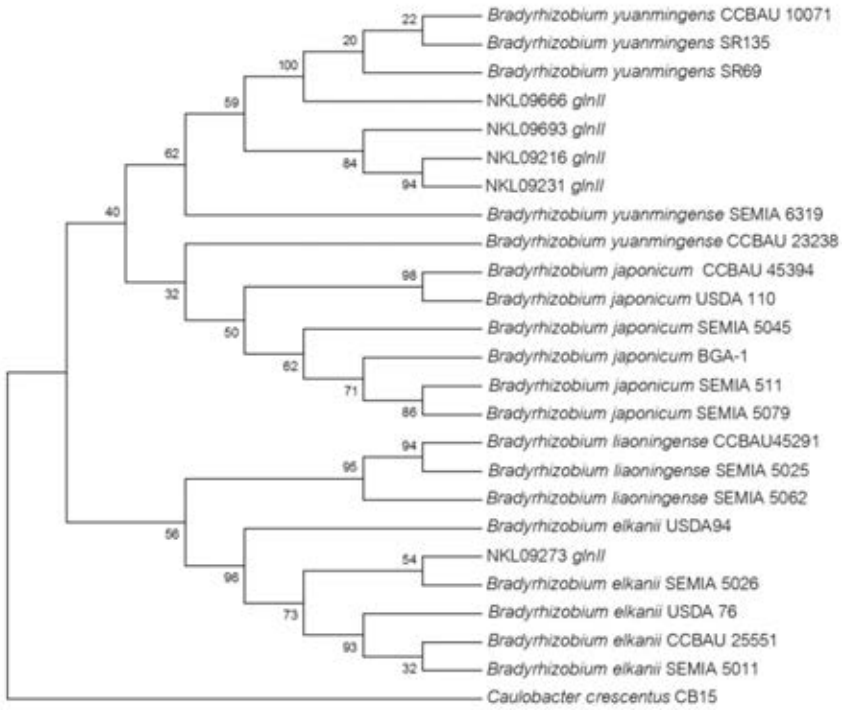


Figure J.3 ML dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of *glnII.*

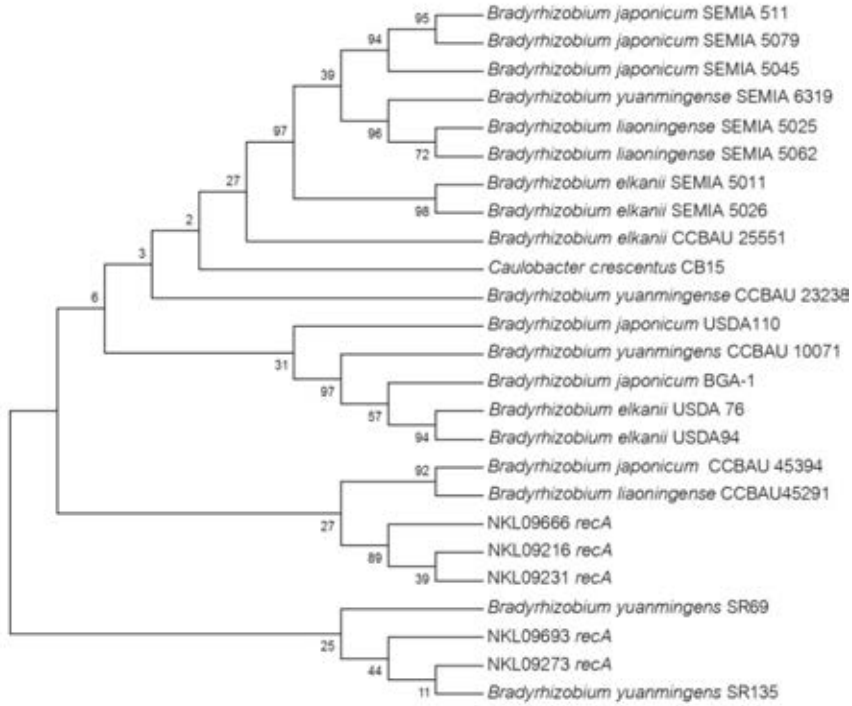


Figure J.4 ML dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of *recA.*

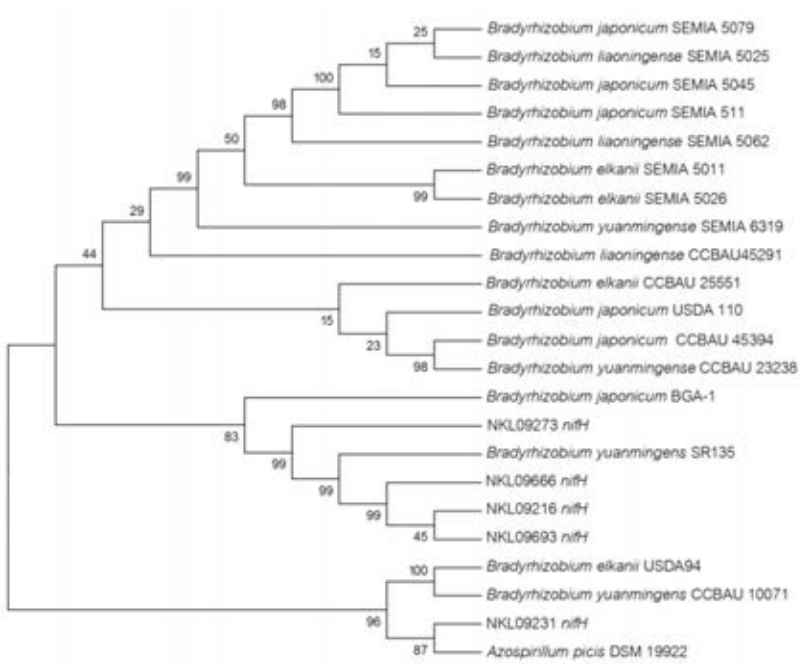


Figure J.5 ML dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of *nifH.*

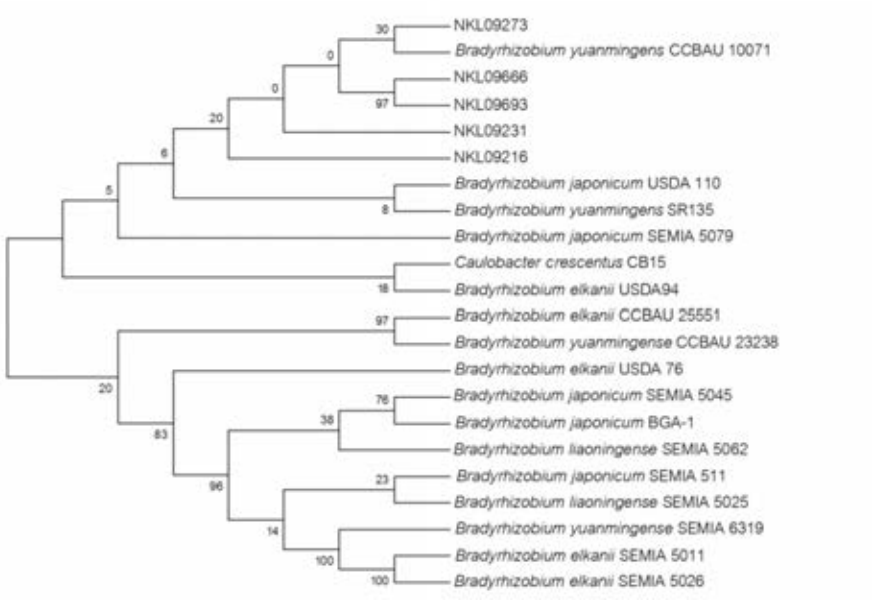


Figure J.6 ML dendrogram constructed from concatenated partial sequences of 16S rDNA-*dnaK- nifH- glnII- recA* of rhizobial strains

NEIGHBOR-JOINING METHOD

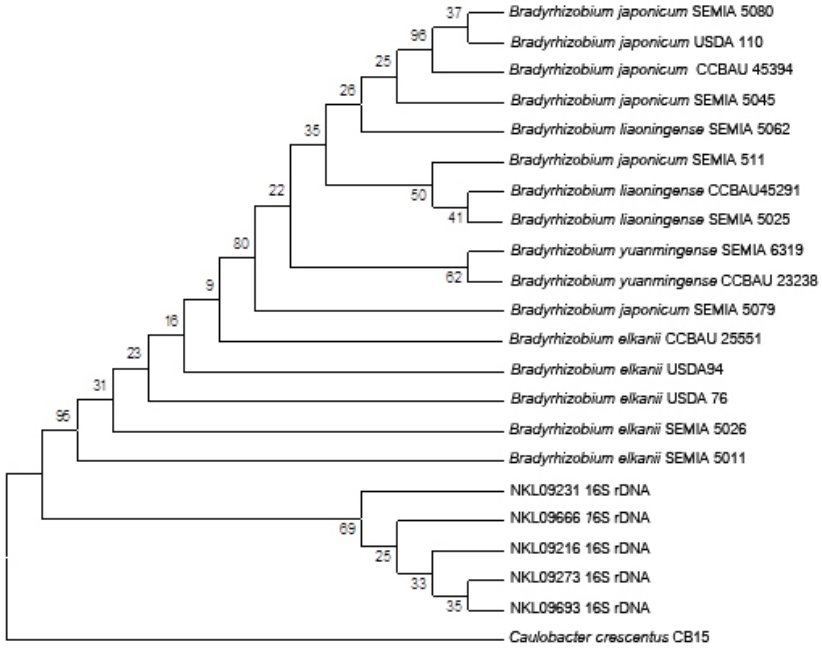


Figure J.7 NJ dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of 16S rDNA.

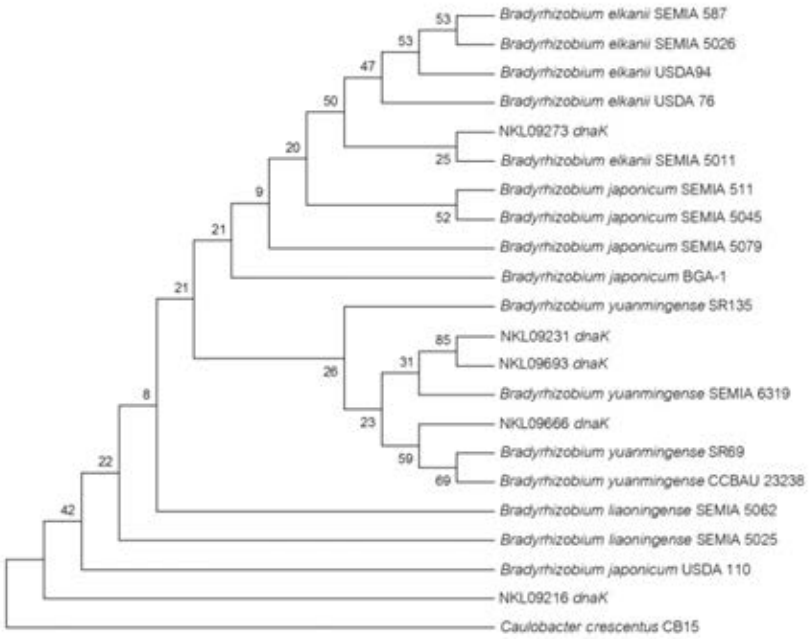


Figure J.8 NJ dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of *dnaK.*

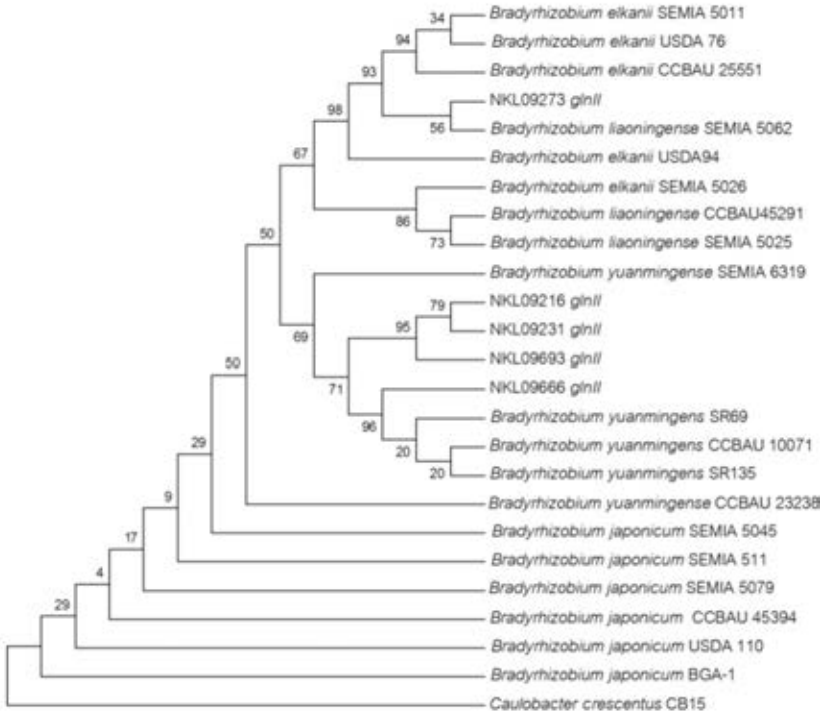


Figure J.9 NJ dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of *glnII.*

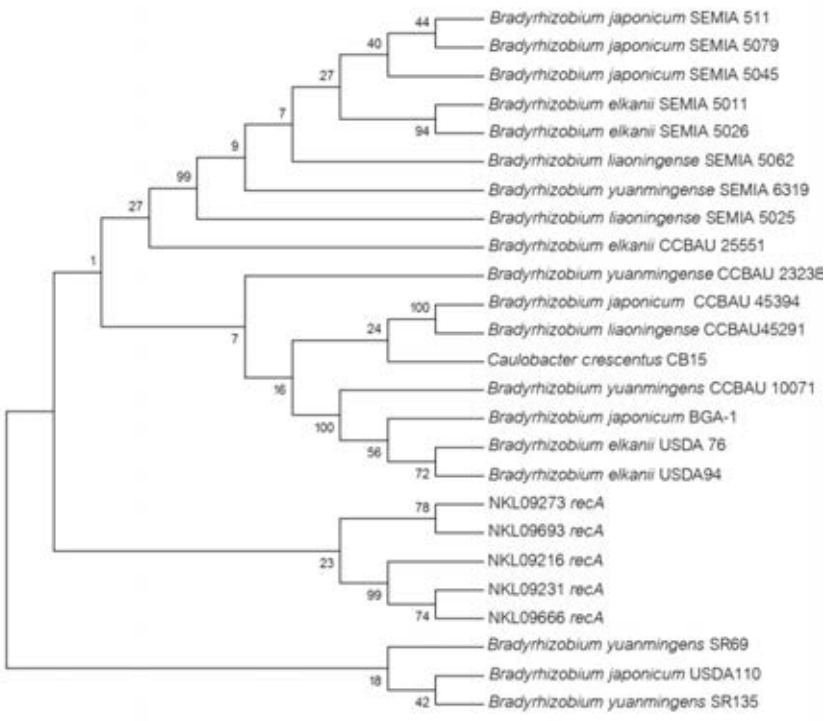


Figure J.10 NJ dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of *recA.*

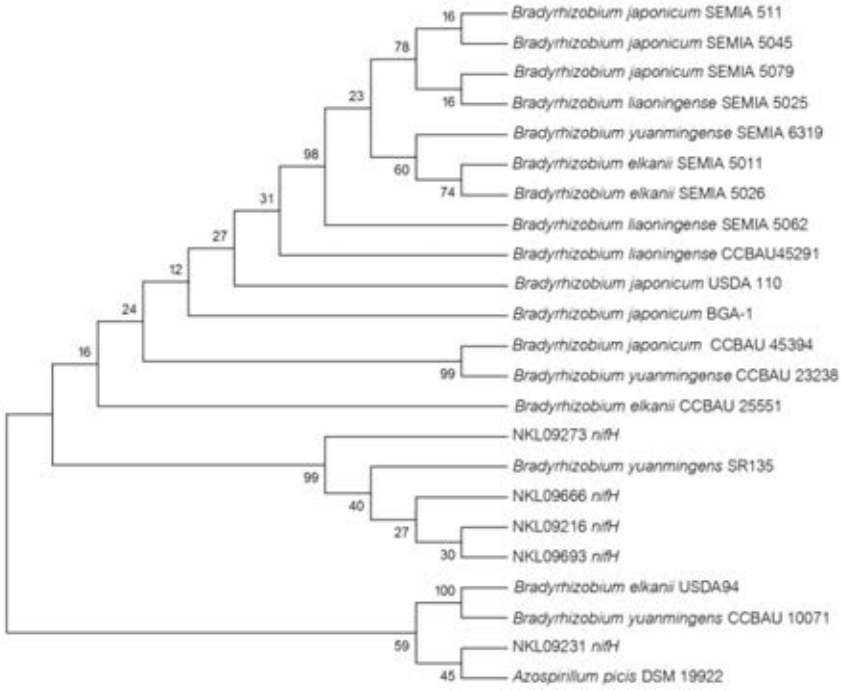


Figure J.11 NJ dendrogram showing relationships amongst soybean rhizobial strains using partial sequences of *nifH.*

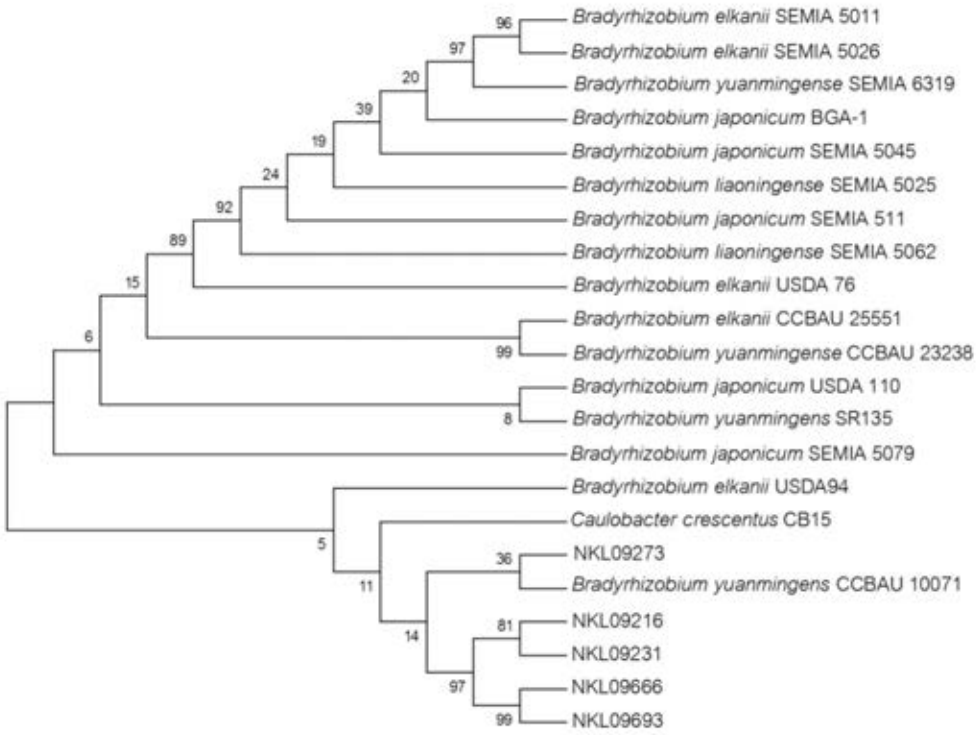


Figure J.12 NJ dendrogram constructed from concatenated partial sequences of 16S rDNA-*dnaK- nifH- glnII- recA* of rhizobial strains

BIOGRAPHY

Miss Yaowapa Punyathiti was born on October 28, 1984. She obtained a Bachelor of Science Degree in Microbiology from Silpakorn University, Sanam Chandra Palace Campus, Nakhon Pathom, Thailand, in 2006.

Publication

1. Punyathiti, Y. and Chansa-ngavej, K. 2012. Genetic diversity of soybean rhizobia at Nong Kula subdistrict, Phitsanulok province. Journal of Science and Technology. 1(2) : 11-18.

2. Sudto, A., Y. Punyathiti and N. Pongsilp. 2008. The use of agricultural wastes as substrates for cell growth and carboxymethyl cellulase (CMCase) production by *Bacillus subtilis*, *Escherichia coli* and *Rhizobium* sp. KMITL Science & Technology Journal. 8(2) : 84-92.

Presentation at Scientific Conference

Yaowapa Punyathiti and Kanjana Chansa-ngavej. 2012. Genetic Diversity of Soybean Rhizobia at Nong Kula Subdistrict, Phitsanulok Province. Abstract Book. The 8th KU-KPS conference : The 8th Kasetsart University Kamphaeng Saen Campus Conference, December 8-9, 2012. Kasetsart University. Nakhon Pathom, Thailand. p 871.