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P Suresh
Assistant Professor, Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India

J Johnson Rajeswar
Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India

K Sukumar
Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India

TJ Harikrishnan
Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India

P Srinivasan
Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India

Correspondence
P Suresh
Assistant Professor, Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India

Complete nucleotide sequence analysis of oncogenicity associated Gene *"pp38"* of Serotype 1 Marek's disease virus isolates from India

P Suresh, J Johnson Rajeswar, K Sukumar, TJ Harikrishnan and P Srinivasan

Abstract
This study was undertaken to characterize the oncogenicity associated *pp38* gene at molecular level for three serotype 1 Marek's Disease Virus (MDV) field isolates recovered from vaccinated poultry flocks which had encountered the outbreak of Marek's Disease (MD) in southern part of India. The prime aim is to generate nucleotide sequence data for important oncogenicity associated *pp38* gene of MDV1 which is very much lacking in India.

Eighty six blood samples were collected from 15 commercial layer and broiler breeder farms. The virus was isolated in duck embryo fibroblasts (DEF) by co - cultivation of lymphocyte and DEF cells. The Isolates were named as Ind/TN/11/01, Ind/KA/12/02 and Ind/ TN/12/03. The oncogenicity associated *pp38* gene was amplified by PCR and sequenced.

The isolates were shown to have a homology of 99.3-99.8 per cent within themselves and 79.9-98.4 per cent between MDVs isolated in this study and other isolates of this area reported by earlier workers for *pp38* gene. The isolates were shown to have a homology of 97.3-98.3 per cent with various isolates of China and 98.4 – 100 per cent with other isolates of Europe and USA. Alignment analysis of the nucleotide sequences had shown nucleotide mutations when MS 53 strain of China was used as reference strain. The nucleotide mutation in the *pp38* gene of MDVs displayed regularity at two positions, including 477 and 640 in the entire field MDV isolates of this study. The mutation at position 477 was unique and coincides with very virulent strains of China and USA including XJ 03, TQ20 and Md5. At position 96 all the isolates are matching with US strains and local isolates of this area reported by earlier workers and differ from MS 53 (G to C). There is a single nucleotide mutation at position 172 (G to C) in the isolate Ind/KA/12/02. There is a single nucleotide mutation at position 548 (G to A) in the isolate Ind/TN/11/01.Phylogenetic analysis on the *pp38* sequence of three isolates and other 10 reference strains showed that the analyzed 13 MDVs could be separated three groups (cluster 1, cluster 2 and cluster 3)

The study implies that the field isolates of serotype 1 MDVs circulating in vaccinated flocks had been shown to have consistent mutations in different positions in *pp38* gene. It would be of use to correlate the *pp38* gene sequence results of this study along with other critical oncogenes viz *Meq* and *vIL8* select a better vaccine candidate.

Keywords: Marek's Disease Virus; Serotype 1; oncogene; *pp38 ;* Nucleotide sequence

Introduction

Biography: The author has got 17 years of professional experience which includes 12 years of teaching and research experience. He had completed his post graduation in the field of Veterinary Microbiology in the year 2000 and acquired the Doctoral degree in 2015 from Tamilnadu Veterinary and Animal Sciences University (TANUVAS). He was appreciated as best PhD student by awarding two gold medals by TANUVAS. He had published 27 research articles in reputed journals including 4 international journals. He had submitted 10 nucleotide sequences at Gen Bank, NCBI. He had published a book on Marek's disease virus and visited countries like France and USA. He is life member in several professional bodies and Institutional bio-safety committees. He has got tremendous interest in teaching to undergraduate students and his unquenchable thirst in teaching had made to become a teaching professional.

Marek's disease (MD) is one of the most common lymphoproliferative diseases of chickens which results in mononuclear cell infiltration of one or more of the visceral organs and nerves (Witter and Schat, 2003). Namakkal poultry belt is the second largest egg bowl in India where the intensity of poultry rearing keeps on increasing resulting in reemergence of Marek's disease. Marek's disease has a tremendous economic impact, firstly because of continuing losses due to the disease and secondly because of cost of vaccination.

Marek's Disease Virus (MDV)is a cell associated herpesvirus. The genome of MDV and the viruses of all three serotypes are broadly similar in their sequences. It consists of a linear, double standard DNA of 160 – 180 kbp in size. The genome consists of unique long (UL) and unique short (US) regions. Several genes unique for MDVs have been identified. Among those, *pp38* gene was reported to have the greatest possibility to be associated with viral oncogenicity and pathogenicity. Therefore, isolation of field MDVs and sequence analysis of *pp38* gene can help us decipher the molecular characters of field MDVs associated with recent epidemics in India. Sequence analysis of field MDVs in India showed that there are virulent MDVs circulating in India (Raja *et al*., 2009; Suresh *et al.,* 2013) in breeder or layer flocks. There is continuous evolution that are taking place in MDV towards greater virulence (Suresh *et al.,* 2017), which has resulted in increased losses from MD in vaccinated flocks (Suresh *et al.,* 2015) now a days. This study is planned to analyze the nucleotide sequences of oncogenecity associated gene *pp38* of the three isolates from flocks which had already been vaccinated with existing monovalent and bivalent vaccines.

Materials and methods

Isolation of Serotype 1 MDV

Samples: Eighty six blood samples were collected from 15 commercial layer and broiler breeder farms throughout Tamilnadu and parts of Karnataka state of India in which MD outbreak occurred in spite of vaccination with monovalent and bivalent vaccines. EDTA was added to blood samples derived from individual farm were pooled to represent that particular farm.

Duck Embryo Fibroblast: Primary cell cultures were prepared from the 10 to 12 day-old embryonated duck eggs as per the protocol of supplemental assay methods (2005), Centre for Veterinary Biologicals, USDA.

Co - cultivation of Lymphocyte and DEF cells: Lymphocytes were collected aseptically from the blood samples which were 132 bp repeats positive (Two or three copies) by using ficoll-paque and co-cultivated with DEF cells as per the method described by Tian *et al*. (2012). The co-cultivated monolayers were observed every day for five to seven days. After three blind passages, the presence of serotype 1 MDV in DEF was verified by PCR detection of 132 bp repeated sequence.

Infecting the Chicken Embryo Fibroblast (CEF) monolayer: The DEF harvests which were positive for two or three copies of 132 bp repeats having no contamination of Avian Leukosis virus (ALV) and Reticuloendotheliosis virus (REV) screened by multiplex PCR kit developed by Gopal *et al*. (2012). The harvests free from ALV and REV were further passaged in CEF monolayer until the appearance of typical MDV plaques. The infected monolayers were observed every day for the formation of plaques upto 7 days.

Amplification of *pp38* gene

DNA extraction: Total DNA was extracted from CEF cells using EZ – Spin Column Viral DNA Mini – Preps Kit (Bio Basic Inc. Canada) and stored at -20ºC

Polimerization chain reaction: Primers for oncogenicity associated *pp38* gene as reported by Tian *et al.* (2011) were synthesized at Bioserve Pvt. Ltd., Hyderabad, supplied in lyophilized form and were used for PCR amplification.The primers were reconstituted in Nuclease free water to get a concentration of 100 pmol/µl (stock) as per manufacturer recommendation. The working concentration of primers is made by diluting the stock solution 1 in 10 to get a concentration of 10 pmol/µl. The primer sequences are shown in table 1.

Table 1: Details of primers for *pp38* gene

S. No.	Primer Sequence	Expected amplicon size (bp).	Targeted gene location	Strain
1.	Forward 5'- TCA TCT TCA ACC CAC AGC CAT CC –3' Reverse 5' – TCG CTT AAT CTC CGC CTC CAA C – 3'	1006 bp	127241-128246	CVI988

PCR was carried out in a final reaction volume of 50 µl using 200 µl capacity thin wall PCR tube. A reaction mixture constitutes Red Dye Master mix (2x) 25 µl, Forward Primers 2 µl, Reverse Primers 2 µl, Template DNA 2 µl and Nuclease free water 19 µl. The PCR protocol was Initial Denaturation at 94ºC for 4 min., Denaturation at 94ºC for 1 min., Annealing at 60ºC for 1 min. Extension at 72ºC for 1.5 min. Final Extension at 72ºC for 10 min. for 35 cycles.

To confirm the targeted PCR amplification, five µl of the PCR product from each tube was loaded and electrophoresed along with 1200bp DNA molecular weight marker (Bio Rad, USA) on 1.5 per cent agarose gel containing ethidium bromide (at the rate of 0.5µg/ml) at constant 80V for 30 min in 0.5X TAE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (BioRad, USA).

Sequencing was done by automated sequencer at Scigenom laboratories, Cochin.

The obtained nucleotide sequences of oncogenic genes of MDV isolates were edited using the Editseq programme in the Lasergene package (DNASTAR Inc, Madison, WI, USA), and compared with other reference MDVs for the homology analysis with the use of MegAlign programme in the same package. Phylogenetic analysis of the nucleotide sequences *pp38* gene was performed with the neighbor-joining method using MEGA version 4.0. The bootstrap values were determined from 1000 replicates of the original data. Nine reference strains were chosen for comparison of *pp38* genes and these MDV reference strains were retrieved from the GenBank database, and the backgrounds of the reference strains used in this study are listed in Table 2.

Table 2: MDV reference strains published in Gen Bank

S. No.	MDV Strain	Virulence	Geographic origin	Year of isolation	Accession number
1.	RB1B	Very virulent	USA	2007	DQ 534541
2.	Md5	Very virulent	USA	1979	AF243438
3.	XJ 03	Very Virulent	China	2011	HQ 638175
4.	JM UA	Virulent	Ukraine	2008	EU771007
5.	TQ 20	Very Virulent	China	2011	HQ 638171
6.	TN0106	Very virulent	India	2009	FJ 620904
7.	TN0207	Very virulent	India	2009	FJ620905
8.	TN0907	Very virulent	India	2009	FJ 620906
9.	TN1207	Very virulent	India	2009	FJ 620907

Results

Isolation

Five representative MDV serotype 1 strains were isolated from 5 of 15 farms by using DEFs culturing (33.33%). Three out of five MDV isolates were free of ALV and REV. They were adapted to CEFs and produced typical cytopathic effect (CPE) (MDV serotype 1 Plaques) after 2-4 passages (Fig.1). The MDV isolates of two farms were discarded because of the contamination of ALV or REV. The isolates were named as Ind/ TN/11/01, Ind/KA/12/02 and Ind/ TN/12/03 respectively.

Amplification of oncogene *pp38*

pp38 gene of all the three isolates was amplified by PCR conditions given in method. All three isolates produced amplicon of size 1006 bp in agarose gel (Fig. 2).

Sequencing of *pp38* gene

The sequences of the MDV isolates of this study are submitted to GenBank and the details are given in table 3.

Table 3: Nucleotide sequences of the isolates submitted in GenBank, NCBI.

S.No.	MDV isolate	Gene	Accession No.
1.	IndTN1203	*pp38*	KP342380
2.	IndTN1101	*pp38*	KP342381
3.	IndKA1202	*pp38*	KP342382

Homology analysis of *pp38* genes between isolates and reference strains

Homology analysis of *pp38* gene showed that the homology of nucleotide sequences within the three isolates were 99.3-99.8 per cent and were 79.9-98.4 per cent between MDVs isolated in this study and other isolates of this area reported by earlier workers. The local isolates were shown to have a homology of 97.3-98.3 per cent with various isolates of China and 98.4 – 100 per cent with other isolates of Europe and USA as shown in Fig 3.

Alignment analysis of *pp38* genes between isolates and reference strains

Alignment analysis of *pp38* complete nucleotide sequences of the three field isolates and 10 published MDVs were performed. Nucleotide mutations were observed when MS 53 strain of China was used as reference strain. The nucleotide mutation in the *pp38* gene of MDVs displayed regularity at two positions, including 477 and 640 in the entire field MDV isolates of this study. The mutation at position 477 was unique and coincides with very virulent strains of China and USA including XJ 03, TQ20 and Md5. At position 96 all the isolates are matching with US strains and local isolates of this area reported by earlier workers and differ from MS 53 (G to C). There is a single nucleotide mutation at position 172 (G to C) in the isolate Ind/KA/12/02. There is a single nucleotide mutation at position 548 (G to A) in the isolate Ind/TN/11/01 (Fig.4)

Phylogenetic analysis of *pp38*

Phylogenetic analysis on the *pp38* sequence of three isolates and other 10 reference strains showed that the analyzed 13 MDVs could be separated three groups (cluster 1, cluster 2 and cluster 3)(Fig.5).

Discussion

Marek's Disease Virus is a member of the genus Mardivirus that consists of serotypes 1 and 2 (MDV-1 and MDV-2) as well as serotype 3 or herpesvirus of turkeys (HVT) (Davison, 2002). Among them, the only serotype 1 is oncogenic, and some of the unique genes such as *Meq* (Jones *et al*., 1992), *pp38* (Cui *et al*., 1991), *vIL8* (viral interleukin-8) (Parcells *et al*., 2001) were reported to be associated with viral oncogenicity and pathogencity. In this study, homology comparison of the nucleotide of *pp38* gene with 9 other reference strains were conducted, and high nucleotide identities of pp38 gene between isolated MDVs and other reference MDVs were studied.

The phosphorylated polypeptide *pp38* of oncogenic MDV is expressed during lytic infections *in vivo* and *in vitro*, but its functions have not been fully elucidated. The *pp38* gene, which codes for 290 aa long protein, is located in the *Bam*HI-H fragment at the junction of UL and IRL regions (Cui *et al*., 1990). The *pp38* antigen is expressed in MDV lymphoblastoid cell lines as well as in tumor samples (Cui *et al*., 1990) and is essential for the maintenance of transformation phenotype of MSB-1 (Xie *et al*., 1996). These findings indicated a role for *pp38* in transformation process. *pp38* also codes a 38kD phosphoprotein and plays a role in viral reactivation in latent period (Yamaguchi *et al*., 2000). Gimeno *et al*., (2005) reported that *pp38* was required to establish cytolytic infection in B lymphocytes but not in FFE, to produce an adequate level of latently infected T cells, and to maintain the transformed status of lymphocytes by preventing apoptosis.

However, the expression of *pp38* is restricted to early cytolytic phase of the MDV infection. Importantly, a direct role of *pp38* only in the early cytolytic infection has been demonstrated by using a *pp38*-null MDV recombinant virus, which did not replicate well but still retained the ability to induce tumors in chickens, albeit at a lower level (Reddy *et al*., 2002). Furthermore, MDV strains belonging to all three serotypes encode for the *pp38*, suggesting that it has no direct role in oncogenesis.

It is contradictory as some workers find the role of *pp38* restricted only to early cytolytic phase and no role in oncogenesis as it is encoded by all the three serotypes. But its role in transformation and reactivation of latency has been proved to an acceptable level. This has led to the idea that *Meq* gene could be a gene of priority rather than *pp38* for

sequence analysis (Tian *et al*., 2011).

Alignment analysis of *pp38* complete nucleotide sequences of the three field isolates of this study and 10 published MDVs were performed. Nucleotide mutations were observed when MS 53 strain (Tian *et al*., 2011) was used as reference strain. The nucleotide mutation in the *pp38* gene of MDVs displayed regularity at two positions, including 477 and 640 occurred in the entire field MDV isolates of this study. The mutation at position 477 was unique and coincides with very virulent strains from China and USA including XJ 03, TQ20, JM UA and Md5. There is a single nucleotide mutation at position 172 (G to C) in the isolate Ind/KA/12/02. There is a single nucleotide mutation at position 548 (G to A) in the isolate Ind/TN/11/01.

MS 53 is a very virulent China strain which is used as reference in this study. Mutation at position 107 and 109 is a character of virulent MDVs (GA, RB1B, 584A and 648A) from USA. But there is no mutation at position 107 in MS 53 and that is similar to isolates of this study. Where as glutamate at position 109 in virulent MDVs (GA, RB1B, 584A and 648A) from USA were replaced by Glycine which is mimicking the changes in field isolates of China (Tian *et al*., 2011). So the mutation (Glutamate to glycine) in position 109 can be considered as a feature of virulent MDVs isolated from USA and China. Single nucleotide mutation at position 477 in *pp38* gene of our isolates can be considered as a feature of field MDVs in southern part of India.

MS 53 is considered very virulent due to one amino acid mutation with USA strains and the mutation at position 477 of the field isolates of this study is considered unique for its virulence and this mutation of local isolates also coincides with very virulent strains of other countries including XJ 03, TQ20 (China) and JM UA and Md5 (USA). At position 96 all the isolates are matching with US strains and local isolates of this area reported by Raja *et al*., (2009) and defers from MS 53 (G to C).

Phylogenetic analysis on the *pp38* sequence of three isolates and other 10 reference strains showed that the analyzed 13 MDVs could be separated into three groups (cluster 1, cluster 2 and Cluster 3). In the cluster 1 the entire local isolates of this study together with very virulent strains from USA including Md5 and RB1B are present along with other isolates of this area by Raja *et al*., (2009). Cluster 2 contains very virulent isolates of China by Tian *et al*. (2011) including XJ03, MS 53 and TQ20. Cluster 2 also contains virulent strain JM along with other isolates of this area by Raja *et al*. (2009).

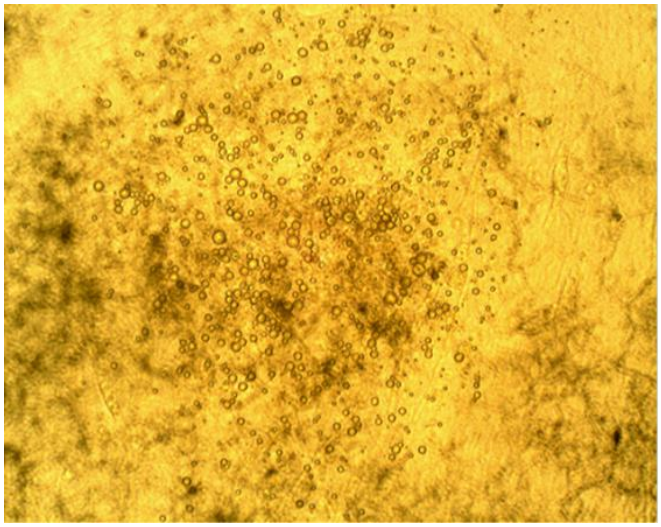


Fig 1: Typical serotype 1 specific MDV plaques induced by isolates in CEF at 2nd passage level

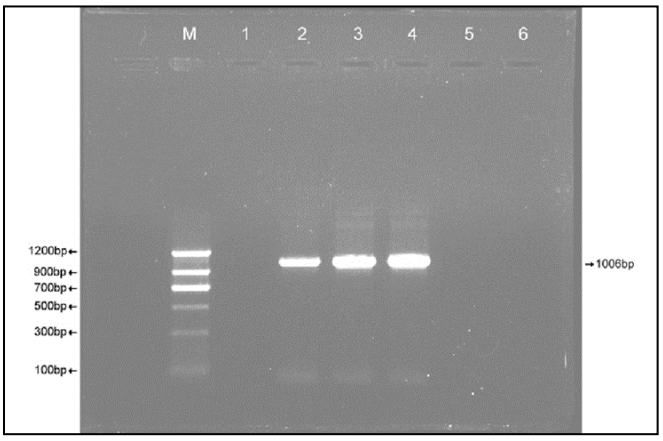


Fig 2: Agarose gel electrophoresis pattern showing amplified PCR product with size of 1006bp of *pp38* gene of the field isolates M-1200bp DNA marker Lane 1 – Non template control Lane 2-4 – Field isolates

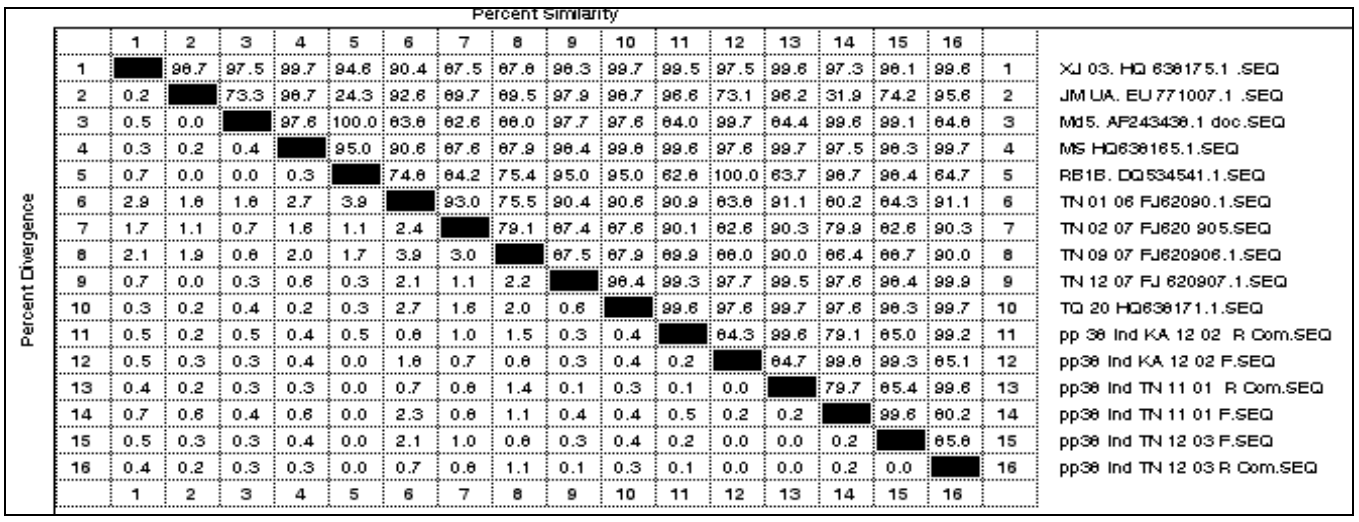
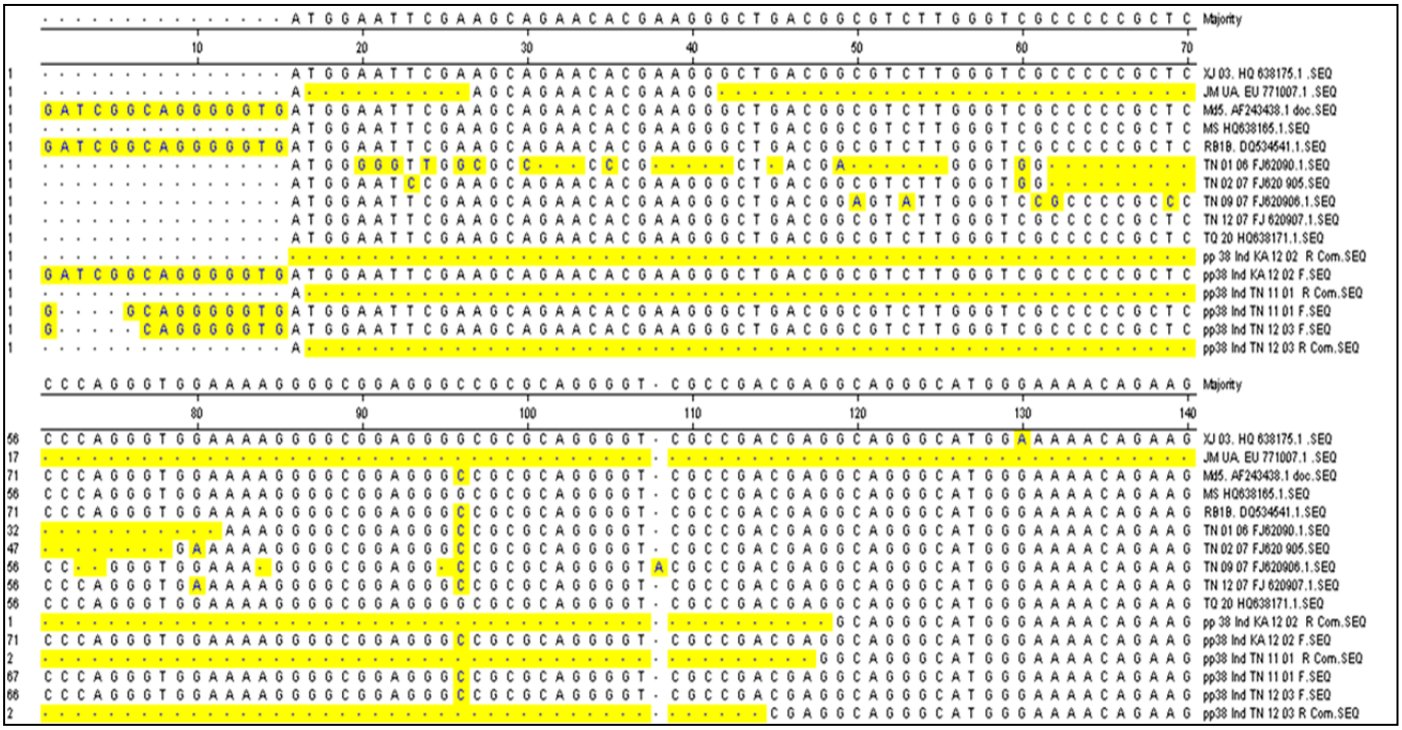


Fig 3: Comparison of homology between the '*pp38*' gene nucleotide sequences of isolates (Ind/TN/11/01, Ind/KA/12/02 & Ind/TN/12/03) and ten other reference sequences in Gen Bank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MS HQ638165.1.SEQ
RB1B. DQ534541.1.SEQ
TN 01 06 FJ62090.1.SEQ
TN 02 07 FJ620 905.SEQ
TN 09 07 FJ620906.1.SEQ
TN 12 07 FJ 620907.1.SEQ
TQ 20 HQ638171.1.SEQ
pp 38 Ind KA 12 02 R Com.SEQ
pp38 Ind KA 12 02 F.SEQ
pp38 Ind TN 11 01 R Com.SEQ
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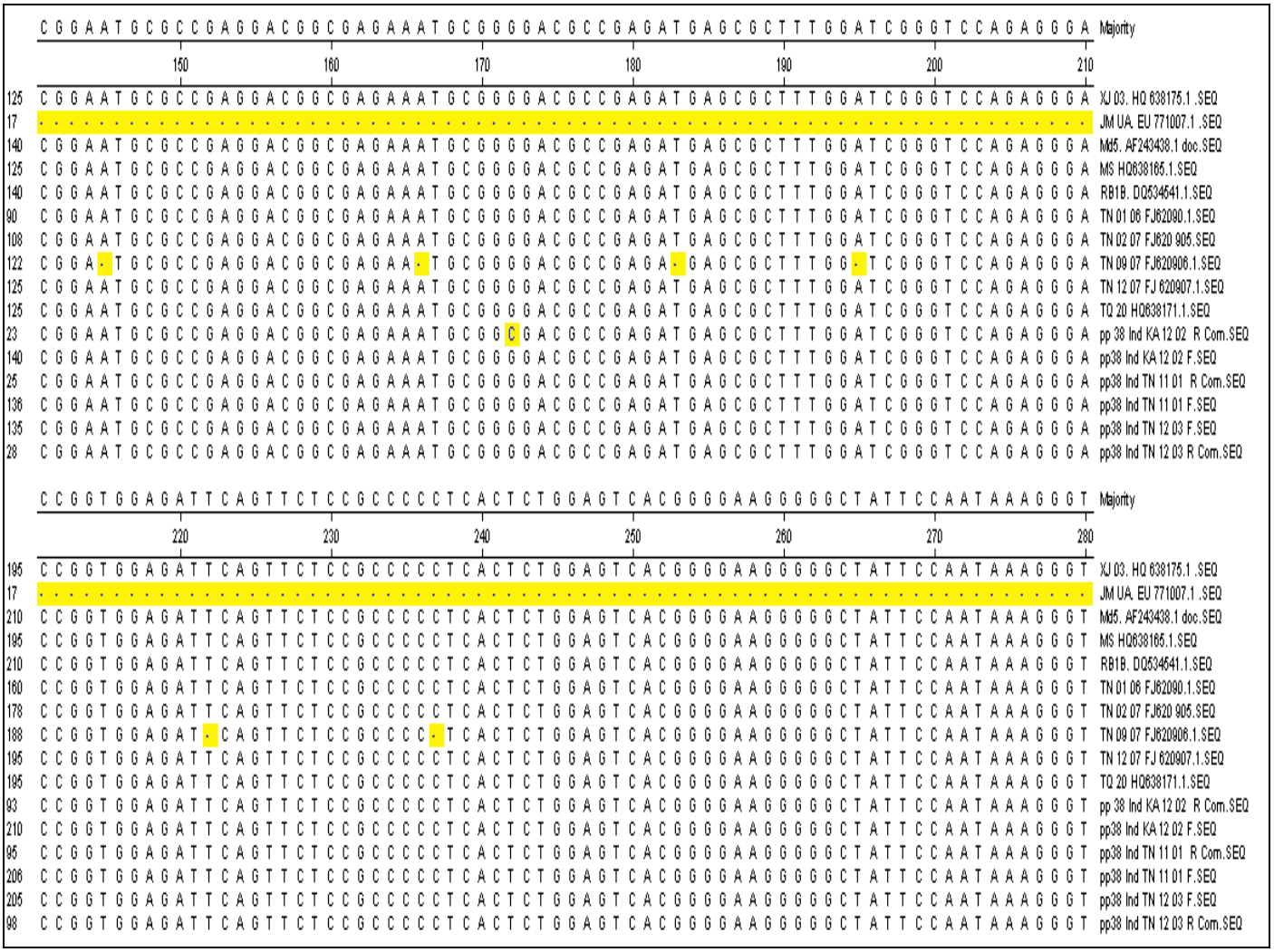
pp38 Ind TN 12 03 F.SEQ
pp38 Ind TN 12 03 R Com.SEQ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TN 02 07 FJ620 905.SEQ
TN 09 07 FJ620906.1.SEQ
TN 12 07 FJ 620907.1.SEQ
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pp 38 Ind KA 12 02 R Com.SEQ
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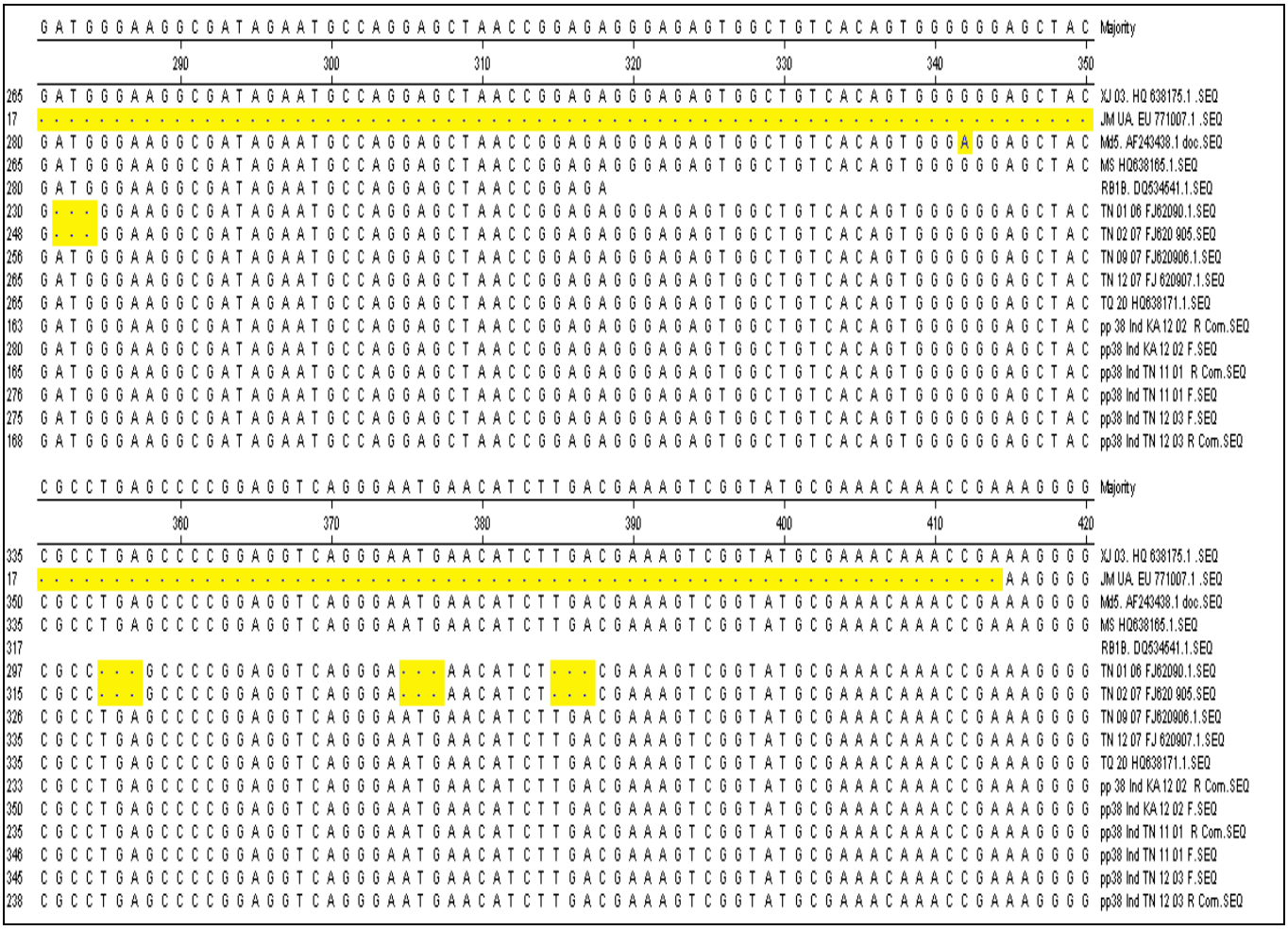
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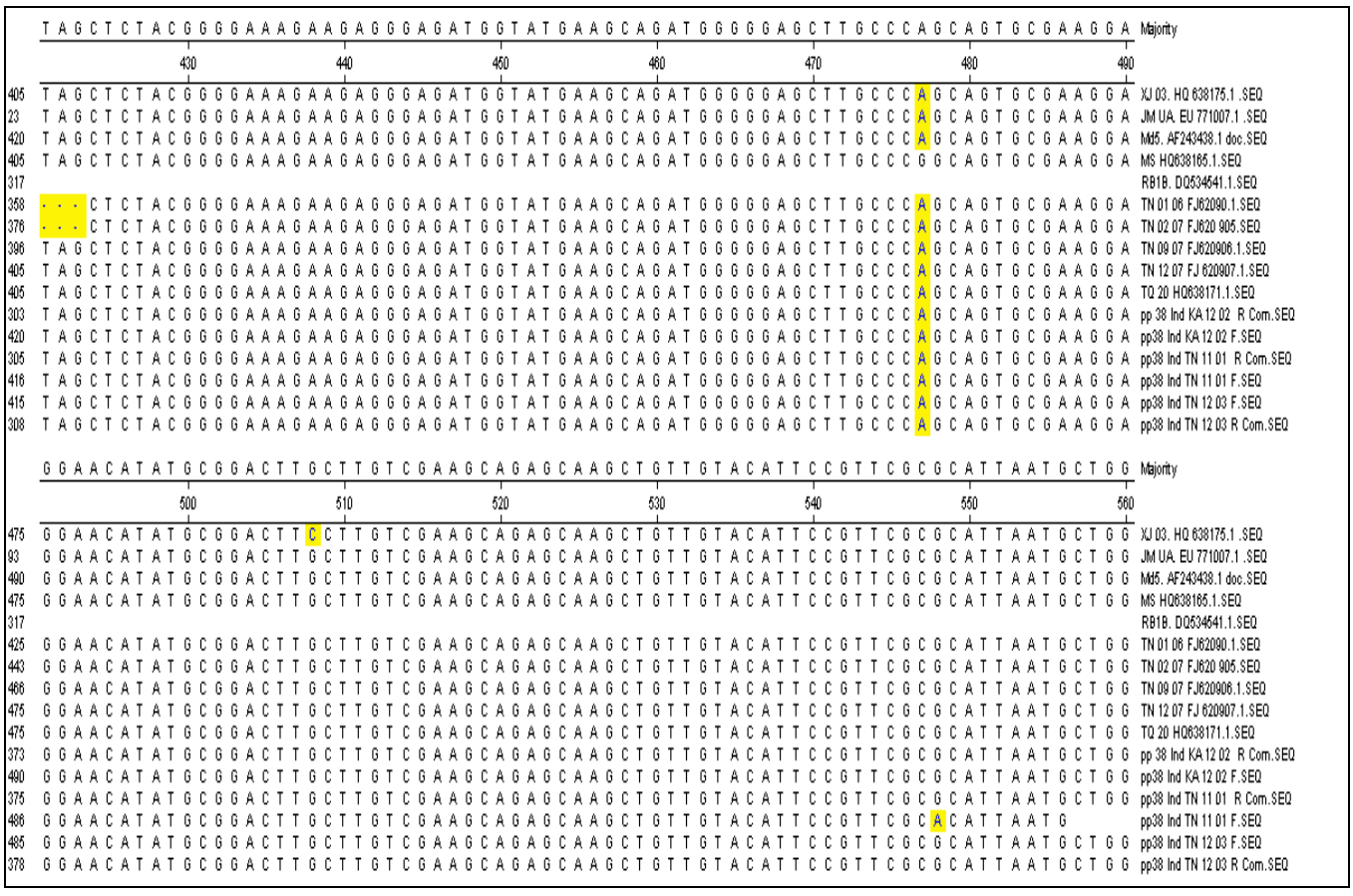
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TQ 20 HQ638171.1.SEQ
pp 38 Ind KA 12 02 R Com.SEQ
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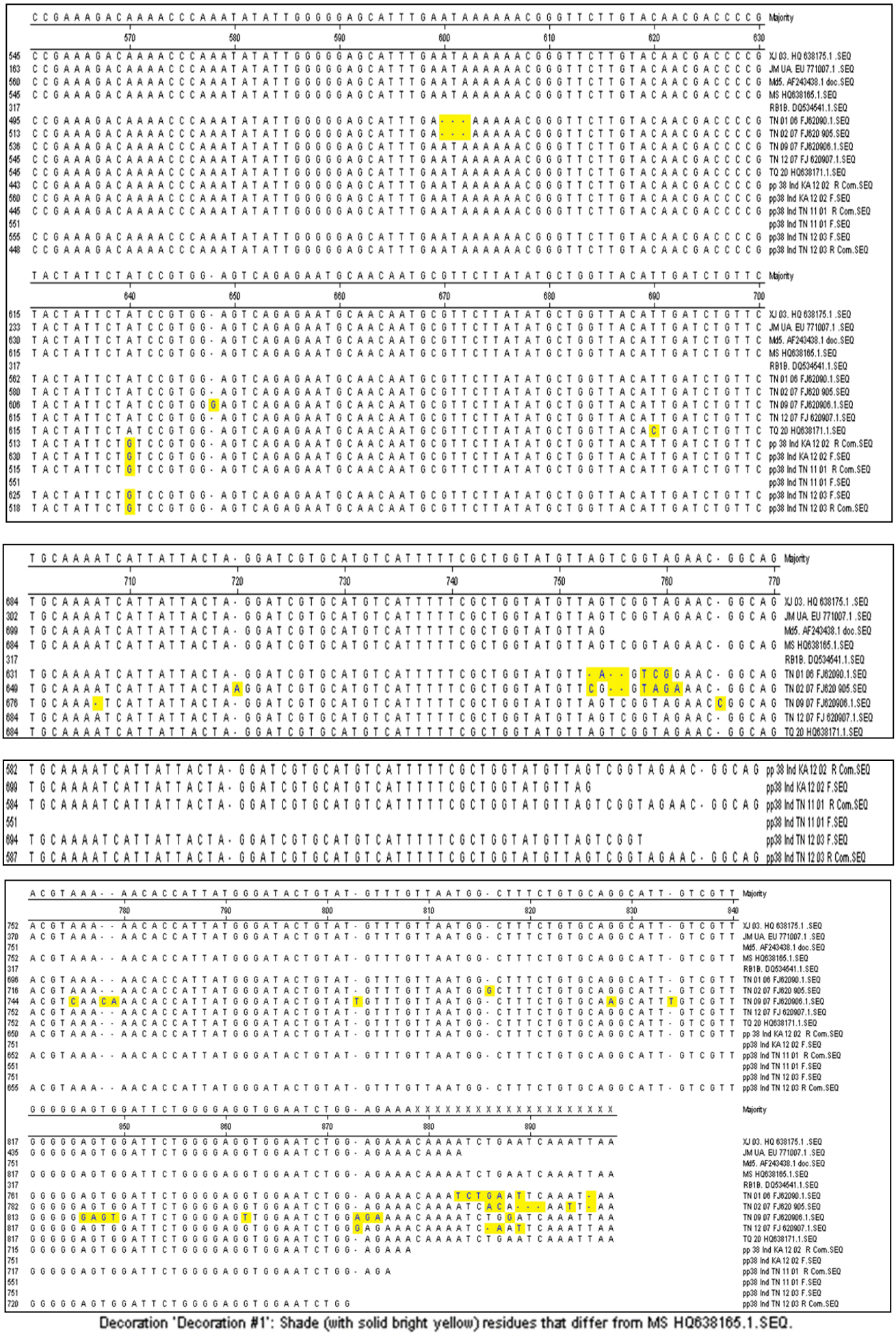

Fig 4: Multiple sequence alignment of a total of 1006 bp nucleotide of '*pp38'* gene for isolates (Ind/TN/11/01, Ind/KA/12/02 & Ind/TN/12/03) in the form of 'CLUSTAL' report with 6 other reference sequences in GenBank

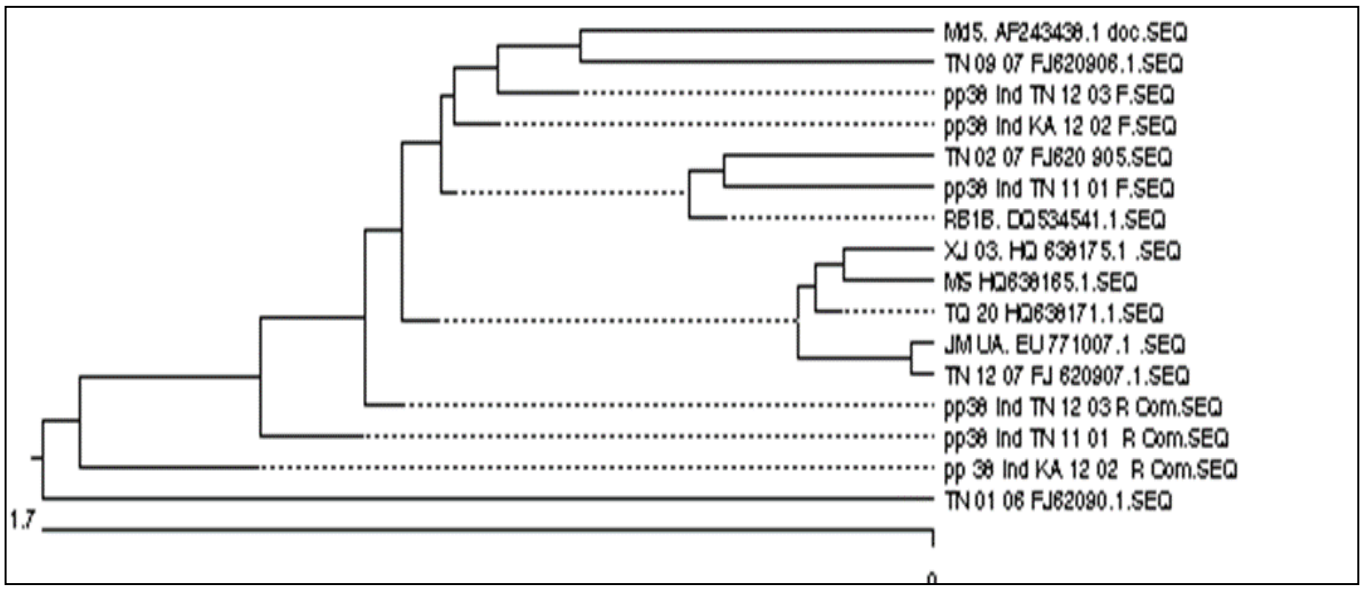

Fig 5: Phylogenetic relationship of isolates (Ind/TN/11/01, Ind/KA/12/02 & Ind/TN/12/03) based on nucleotide sequences of '*pp38*' genes with ten other reference sequences in GenBank

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