RESULTS

The polymerase chain reaction–restriction fragment length polymorphism (**PCR-RFLP**) technique was used to detect the effect of malathion on butyrylcholinesterase (BChE) gene of mice embryo. The restriction fragments of PCR-RFLP which have been separated by agarose gel electrophoresis were obtained as photos to identify the gene mutations.

The total genomic DNA of normal and treated samples is represented in **Fig. (1)**. Lane M represents the DNA marker (100-2000 bp). While lanes 1 to 4 represent the DNA genome of normal, 1/100, 1/60 and 1/30 samples, respectively. Whereas, PCR products of BChE gene of normal and treated samples are found in, **Fig. (2)**. The size of BChE gene product was approximately 1812 bp in normal and treated samples (**Fig., 2**).

Ten restriction enzymes were used in RFLP technique for the digestion of BChE gene of normal and treated samples. In addition, three programs (Webcutter v. 2.0, Bioface & Gblocks v. 0.91b) were used for the analysis of RFLPs patterns and predicating mutations positions and values. According to RFLPs profiles, *Alw*I, *Bpi*I and *Nsi*I enzymes could not differentiate between normal (**Lane, 1**) and treated groups (1/100, **Lane, 2**; 1/60, **Lane, 3** and 1/30, **Lane, 4**). *Nsi*I restriction enzyme digested BChE gene of all samples into two bands at lengths 412 bp and 1400 bp (**Fig., 3 & 13 and Table, 1**). While, *Alw*I endonuclease enzyme restricted BChE gene of all samples into four patterns with lengths 327 bp, 426 bp, 467 bp and 592 bp (**Fig., 4 & 14 and Table, 2**). Moreover, *Bpi*I enzyme cut BChE gene of all studied samples into four fragments with lengths 96 bp, 246 bp, 264 bp and 1206 bp (**Fig., 5 & 15 and Table, 3**).

The rest of restriction enzymes distinguished between normal and treated samples. In addition, they almost differentiated between the treated individuals. *BstB*I restriction enzyme clustered the gene of all samples into two clusters (**Table, 4 and Fig., 6 & 16**). The first cluster which includes the gene of normal sample was restricted into three fragments at lengths 463 bp, 470 bp and 879 bp (**Lane, 1**). While the second cluster which contains the gene of 1/100, 1/60 and 1/30 samples was cut into four bands at lengths 365 bp, 463 bp, 470 bp and 514 bp (**Lanes, 2; 3 & 4**). As a result it could be predicted that the gene of all treated groups has the same mutation at position 518 where the nucleotide T changed into A (TTCGA**T**→TTCGA**A**, **Fig., 23**). Accordingly, there is a change in the amino acid M (Methionine) into K (Lysine) at position 173 of all treated groups protein (**Fig., 24 and Table, 11**). Consequently, *BstB*I enzyme differentiated the normal group.

The results of BChE gene digestion by *BspD*I restriction enzyme divided the samples into two sums (**Table, 5 and Fig., 7 & 17**). The first sum showed the digestion of normal, 1/100 and 1/60 samples into two bands at lengths 456 bp and 1356 bp (**Lanes, 1; 2 & 3**). Whereas, the second sum demonstrated that 1/30 sample was cut into three fragments at lengths 456 bp, 514 bp and 842 bp (**Lane, 4**). From this it could be indicated that a mutation occurred in 1/30 gene where the nucleotide T changed into A (**T**TCGAT→**A**TCGAT) at position 513 (**Fig., 23 and Table, 11**) generating no change in the amino acid of BChE protein (silent mutation). Thus *BspD*I distinguished 1/30 sample.

All samples were cut with *Sph*I endonuclease enzyme and differentiated into two groups (**Table, 6 and Fig., 8 & 18**). The gene of normal, 1/100 and 1/60 samples group was digested into three patterns with lengths 66 bp, 75 bp and 1671 bp (**Lanes, 1; 2 & 3**). While 1/30 sample was restricted into four bands at lengths 66 bp, 75 bp, 410 bp and 1261 bp (**Lane, 4**). Therefore, it could

be indicated that a mutation occurred in 1/30 gene in which the nucleotide G changed into C (GCATG**G**→GCATG**C**, **Fig., 23)** at position 1403. This nucleotide change transformed the amino acid G (Glutamine) into A (Alanine) at position 468 of the 1/30 BChE protein (**Fig., 24 and Table, 11**). So *Sph*I enzyme differentiated 1/30 sample.

*Hae*III restriction enzyme grouped the gene of all samples into two groups (**Table, 7 and Fig., 9 & 19**). Normal, 1/100 and 1/60 samples group was digested into four fragments at lengths 180 bp, 243 bp, 255 bp and 1134 bp (**Lanes, 1; 2 & 3**). Whereas the gene of 1/30 sample group was restricted into only three bands at lengths 243 bp, 435 bp and 1134 bp (**Lane, 4**). Consequently, it could be predicted that the gene of 1/30 sample might have one or more mutations where the nucleotides G and C could be changed at positions 1556 and/or 1557 and/or 1558 and/or 1559 (**Fig., 23 and Table, 11**). Accordingly, the amino acids W (Tryptophan) at position 519 and/or P (Proline) at position 520 of 1/30 BChE protein could be changed into another types of amino acids (**Fig., 24 and Table, 11**). Therefore, *Hae*III restriction enzyme distinguished 1/30 group.

The digestion of all samples by *Mae*II endonuclease enzyme has been differentiated into two clusters (**Table, 8 and Fig., 10 & 20**). The gene of normal and 1/100 samples cluster was cut by this restriction enzyme into two cuts at lengths 607 bp and 1205 bp (**Lanes, 1 & 2**). On the other hand the gene of 1/60 and 1/30 samples cluster was digested into three patterns at lengths 285 bp, 607 bp and 920 bp (**Lanes, 3 & 4**). As a result it could be predicted that the gene of both 1/60 and 1/30 samples has the same mutation at position 920 where the nucleotide T changed into A (**T**CGT→**A**CGT, **Fig., 23**). Consequently, the amino acid F (Phenylalanine) modified into Y (Tyrosine) at position 307 of 1/60 and 1/30 BChE protein (**Fig., 24 and Table, 11**).

The gene of all samples was cut by *Sca*I restriction enzyme resulting in three clusters (**Table, 9 and Fig., 11 & 21**). The normal gene cluster was digested into two fragments at lengths 240 bp and 1572 bp (**Lane, 1**). Whereas, 1/100 gene cluster was restricted into three bands at lengths 240 bp, 389 bp and 1183 bp (**Lane, 2**). Moreover, the cluster of 1/60 and 1/30 samples was digested into four patterns at lengths 240 bp, 389 bp, 568 bp and 615 bp (**Lanes, 3 & 4**). Thus it could be predicted that the gene of 1/100 gene has a mutation at position 392 where the nucleotide C changed into T (AGTAC**C**→ AGTAC**T**, **Fig., 23**), also, the amino acid P (Proline) changed into L (Leucine) at position 131 (**Fig., 24 and Table, 11**) of 1/100 BChE protein. While, the gene of both 1/60 and 1/30 samples has the same mutation like 1/100 sample as well another mutation at position 1003 where the nucleotide C changed into G (A**C**TACT→ A**G**TACT, **Fig., 23**) which indicate a change in the amino acid L (Leucine) into V (Valine) at position 335 (**Fig., 24 and Table, 11**) of 1/60 and 1/30 BChE proteins.

The gene of all samples was cut by *Dra*I restriction enzyme giving three sums (**Table, 10 and Fig., 12 & 22**). The first sum (normal and 1/100 samples) was restricted into two fragments with lengths 664 bp and 1148 bp (**Lanes, 1 & 2**). Whereas, the gene of the second sum (1/60 sample) was digested into three bands with lengths 42 bp, 622 bp and 1148 bp (**Lane, 3**) and the gene of the last sum (1/30 sample) was cut into four patterns with lengths 42 bp, 233 bp, 622 bp and 915 bp (**Lane, 4**). Consequently, it could be indicated that the gene of 1/60 has one mutation at position 1773 where the nucleotide C changed into A (TTTAA**C**→TTTAA**A**, **Fig., 23**) which resulted in the change of the amino acid N (Asparagine) into K (Lysine) at position 591 of 1/60 BChE protein (**Fig., 24 and Table, 11**). Also, the gene of 1/30 sample has the same mutation as 1/60 sample besides another mutation at position 231 (**Table, 11**) where the

nucleotide C changed into T (**C**TTAAA→**T**TTAAA, **Fig., 23**) giving a silent mutation in 1/30 BChE protein.

According to **table (12)**, the normal gene nucleotide sequence (1812 bp) contains 539 bases of **A**, 487 bases of **T**, 383 bases of **C** and 403 bases of **G**. Therefore, (**A + T)** content is 1026 (56.6%) and (**C + G)** content is 786 (43.4%). While the 1/100 gene sequence has 540 **A** bases and one 382 **C** bases which increase (**A + T)** content into 1027 and decrease (**C + G)** content into 785 rather than normal sequence. This change in the nucleotides values is obtained using *BstB*I & *Sca*I restriction enzymes. Also, the values of 1/60 BChE gene nucleotides has been altered as a result of digestion by *Mae*II, *BstB*I, *Dra*I, *Sca*I & *Sca*I endonucleases. **A** bases increased into 542, **T** bases decreased into 486, **C** bases declined into 380 and **G** bases raised into 404. Consequently, (**A + T)** content increased into 1028 and (**C + G)** content decreased into 784 when compared to the normal gene. Furthermore, the values of nucleotides in 1/30 gene has been changed where **A** bases rose into 543, **T** bases declined into 486 and **C** bases declined into 380. According to this, (**A + T)** content increased into 1029 and (**C + G)** content decreased into 783 rather than the normal gene. All the above changes in 1/30 BChE gene is accomplished after digestion with *BspD*I, *Mae*II, *BstB*I, *Sph*I, *Dra*I, *Sca*I *&* *Sca*I enzymes. However, by using *Hae*III restriction enzyme with 1/30 gene, it is appeared that the values of **A**, **T**, **C** and **G** bases are altered but without knowing the exact values of the nucleotides variations.

In addition, **table (13)** shows that the amino acids values of 1/100 BChE protein is different than the normal protein including Leucine (55→56), Lysine (35→36), Methionine (18→17) and Proline (37→36). In addition, the values of amino acids of 1/60 protein is altered rather than normal group as a result of variations in Asparagine (33→32), Lysine (35→36), Methionine (18→17),

Phenylalanine (40→39), Proline (37→36), Tyrosine (22→23) and Valine (33→34). Moreover, there are changing in Alanine (35→36), Asparagine (33→32), Glycine (46→45), Lysine (35→36), Methionine (18→17), Phenylalanine (40→39), Proline (37→36), Tryptophan (16→15), Tyrosine (22→23) and Valine (33→34) in 1/30 group. Accordingly, 1/30 protein is altered greatly in its amino acids' values when compared to normal protein.

Fig. (25) shows the Phylogenetic distance tree obtained with the Neighbor Joining method (using Geneious software v. 4.8) applied to the studied groups gene sequences in **Fig. (23**). This cladogram is predicated that, these groups are divided into two clusters. The first cluster includes normal and 1/100 groups that convened at a node height of 0.00333 (0.33%) s/s (substitution per site) and separated from each other by 0.00111 (0.11%) s/s. However, the second cluster contains 1/60 and 1/30 groups which gather at a node height of 0.00167 (0.167%) s/s and separated from each other by 0.00167 (0.167%) s/s. Additionally, the distance between the previous two clusters is 0.00166 (0.166%) s/s. Moreover, it is appeared from the distance matrix (**Table, 14**), that 1/100, 1/60 and 1/30 groups are separated from the normal group by 0.00111 (0.11%) s/s, 0.00277 (0.28%) s/s and 0.00444 (0.44%) s/s, respectively. Furthermore, 1/100 and 1/60 groups are separated from 1/30 group by 0.00333 (0.33%) s/s and 0.00166 (0.166%) s/s, respectively.

According to RNA2 prediction software (v 2.0), the number of stems of the treated groups (1/100, 1/60 & 1/30) RNA secondary structures (**Fig., 27; 28 & 29. Table, 15**) is different than the normal group (**Fig., 26. Table, 15**). The normal group contains 65 stems while 1/100, 1/60 and 1/30 groups have 62, 63 and 61 stems, respectively with free energies ranging from -6.1 kcal/mol to -22.5 kcal/mol and nucleotide sequences ranging between 3 bp and 13 bp for all groups. In addition, the free energy of the normal group RNA secondary

structure is -338.7 kcal/mol whereas, the free energies of 1/100, 1/60 and 1/30 RNA secondary structures are -338.6 kcal/mol, -342.8 kcal/mol and -325.8 kcal/mol, respectively.

By comparing the three treated groups (1/100, 1/60 & 1/30) with the normal one (**Table, 15**), the sequences of 6, 11, 14, 15, 16, 20, 24, 25, 41, 47, 54, 55, 56, 59, 60, 62, 63 and 64 stems are uniquely found in normal group. The sequences of stems 32 and 34 of normal group are similar to 1/100 group only and sequences of stems 27, 38 and 58 of normal group are alike to 1/100 and 1/60 groups only. Additionally, the sequence of stem 17 of normal group (starts at position 262) is the same of 1/30 group (starts at position 409) only. Also, the sequence of stem 30 of normal group (begins at position 761) is found in 1/60 and 1/30 groups (begins at position 970) only. Moreover, the sequence of stem 37 of normal group is present in all groups starting at the same position of 246 except in 1/30 group where this sequence starts at position 209.

Additionally, there are some stems created uniquely in the treated groups when compared with the normal one (**Table, 15**) such as 6, 7, 13 and 15 stems. Also, the sequences of stems 18, 26, 31, 45, 48, 49, 56, 60 and 61 of 1/100 group are present in the other two treated groups only. In addition, the sequences of stems 20, 21 and 40 of 1/100 group are the same of 1/60 group only. Moreover, sequences of 45, 47 and 49 stems of 1/60 group are present in 1/30 group only. Furthermore, the sequence of stem 24 is uniquely found in 1/100 group and the sequences of stems 31, 43 and 45 are uniquely present in 1/30 group.

Fig. (1): Represents the total genomic DNA of normal and treated samples. Lane M is the DNA marker (100-2000 bp) while lanes 1 to 4 symbolize the DNA genome of normal, 1/100, 1/60 and 1/30, respectively.

Fig. (2): Shows the PCR products of normal and treated samples. Lane M is the DNA marker (50-2000 bp) while lanes 1 to 4 represent the PCR products of BChE gene of normal, 1/100, 1/60 and 1/30, respectively. The size of BChE gene was 1812 bp in all samples.

Fig. (3): Shows the fragments resulted after the digestion of BChE gene of all groups by *Nsi*I restriction enzyme. Lane M is the DNA marker while lanes 1 to 4 represent the fragments of normal, 1/100, 1/60 and 1/30, respectively.

Fig. (4): Shows the fragments resulted after the digestion of BChE gene of all groups by *Alw*I restriction enzyme. Lane M is the DNA marker while lanes 1 to 4 represent the fragments of normal, 1/100, 1/60 and 1/30, respectively.

Fig. (5): Shows the fragments resulted after the digestion of BChE gene of all groups by *Bpi*I restriction enzyme. Lane M is the DNA marker while lanes 1 to 4 represent the fragments of normal, 1/100, 1/60 and 1/30, respectively.

Fig. (6): Shows the fragments resulted after the digestion of BChE gene of all groups by *BstB*I restriction enzyme. Lane M is the DNA marker while lanes 1 to 4 represent the fragments of normal, 1/100, 1/60 and 1/30, respectively.

Fig. (7): Represents the fragments resulted after the digestion of BChE gene of all groups by *BspD*I restriction enzyme. Lane M is the DNA marker while lanes 1 to 4 represent the fragments of normal, 1/100, 1/60 and 1/30, respectively.

Fig. (8): Shows the fragments resulted after the digestion of BChE gene of all groups by *Sph*I endonuclease enzyme. Lane M is the DNA marker while lanes 1 to 4 represent the fragments of normal, 1/100, 1/60 and 1/30, respectively.

Fig. (9): Shows the fragments resulted after the digestion of BChE gene of all groups by *Hae*III restriction enzyme. Lane M is the DNA marker while lanes 1 to 4 represent the fragments of normal, 1/100, 1/60 and 1/30, respectively.

Fig. (10): Represents the fragments resulted after the digestion of BChE gene of all groups by *Mae*II endonuclease enzyme. Lane M is the DNA marker while lanes 1 to 4 represent the fragments of normal, 1/100, 1/60 and 1/30, respectively.

Fig. (11): Shows the fragments resulted after the digestion of BChE gene of all groups by *Sca*I restriction enzyme. Lane M is the DNA marker while lanes 1 to 4 represent the fragments of normal, 1/100, 1/60 and 1/30, respectively.

Fig. (12): Shows the fragments resulted after the digestion of BChE gene of all groups by *Dra*I restriction enzyme. Lane M is the DNA marker while lanes 1 to 4 represent the fragments of normal, 1/100, 1/60 and 1/30, respectively.

Table (1): Represents the two fragments lengths of BChE gene which were restricted by *Nsi*I enzyme in all the studied groups.

Bands / Groups	Band#1	Band#2	Band#3	Band#4
Normal	412	1400	----------	----------
1/100	412	1400	----------	----------
1/60	412	1400	----------	----------
1/30	412	1400	----------	----------

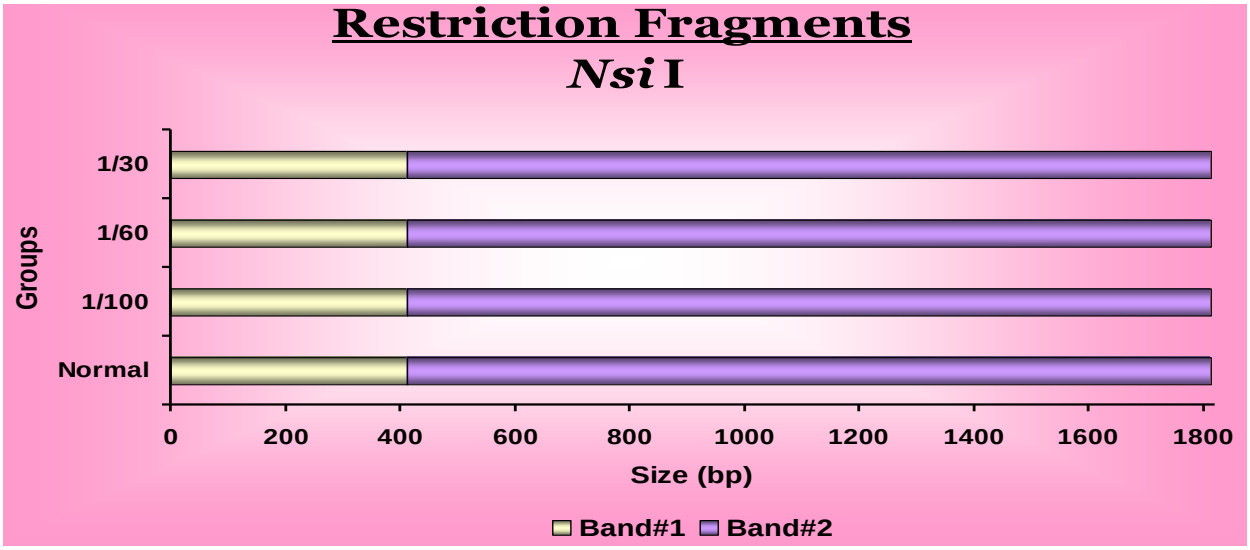

Fig. (13): Shows the two fragments lengths of BChE gene which were restricted by *Nsi*I enzyme in all the studied groups.

Table (2): Shows the four patterns lengths of BChE gene which were cut by *Alw*I restriction enzyme in the studied groups.

Bands / Groups	Band#1	Band#2	Band#3	Band#4
Normal	327	426	467	592
1/100	327	426	467	592
1/60	327	426	467	592
1/30	327	426	467	592

Fig. (14): Represents the four patterns lengths of BChE gene which were cut by *Alw*I restriction enzyme in the studied groups.

Table (3): Shows the four bands lengths of BChE gene resulted from digestion by *Bpi*I endonuclease in the studied samples.

Bands / Groups	Band#1	Band#2	Band#3	Band#4
Normal	96	246	264	1206
1/100	96	246	264	1206
1/60	96	246	264	1206
1/30	96	246	264	1206

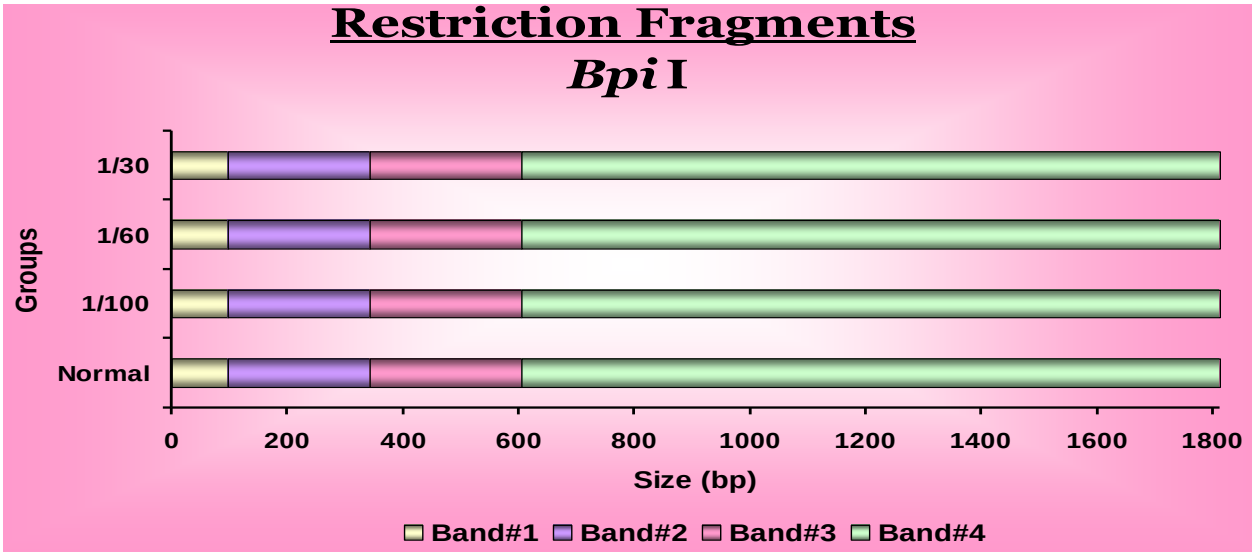

Fig. (15): Represents the four bands lengths of BChE gene resulted from digestion by *Bpi*I endonuclease in the studied samples.

Table (4): Represents the bands lengths of BChE gene predicated from restriction with *BstB*I enzyme in the studied groups.

Bands / Groups	Band#1	Band#2	Band#3	Band#4
Normal	463	470	879	----------
1/100	365	463	470	514
1/60	365	463	470	514
1/30	365	463	470	514

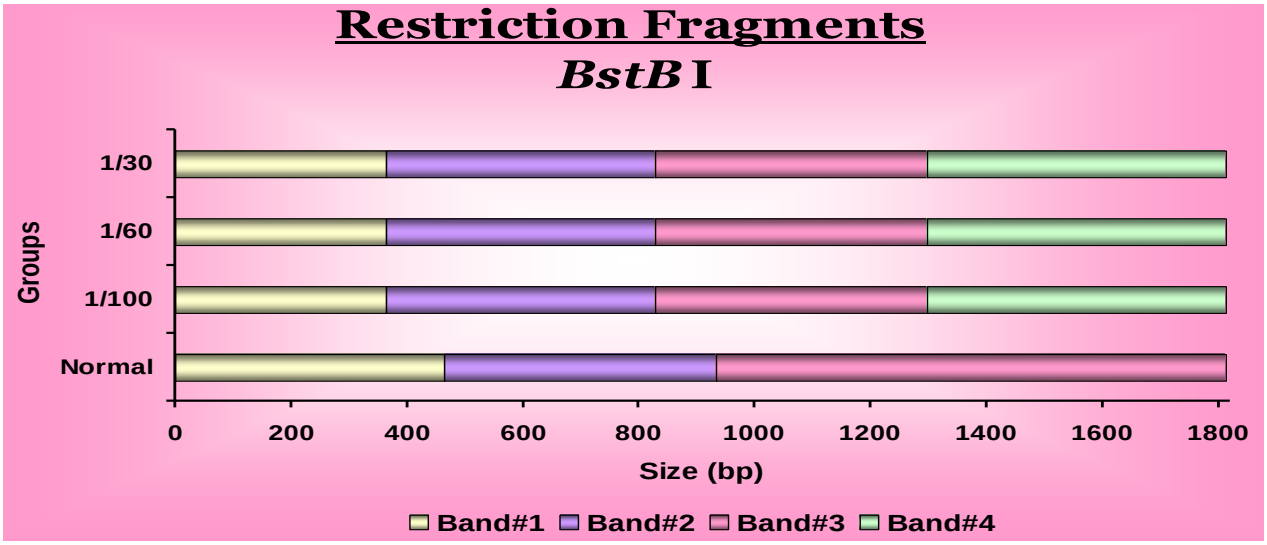

Fig. (16): Shows the bands lengths of BChE gene predicated from restriction with *BstB*I enzyme in the studied groups. Normal group is digested into three bands while the treated groups are digested into four bands.

Table (5): Represents the fragments lengths of BChE gene predicated from restriction by *BspD*I enzyme in the studied samples.

Bands / Groups	Band#1	Band#2	Band#3	Band#4
Normal	456	1356	----------	----------
1/100	456	1356	----------	----------
1/60	456	1356	----------	----------
1/30	456	514	842	----------

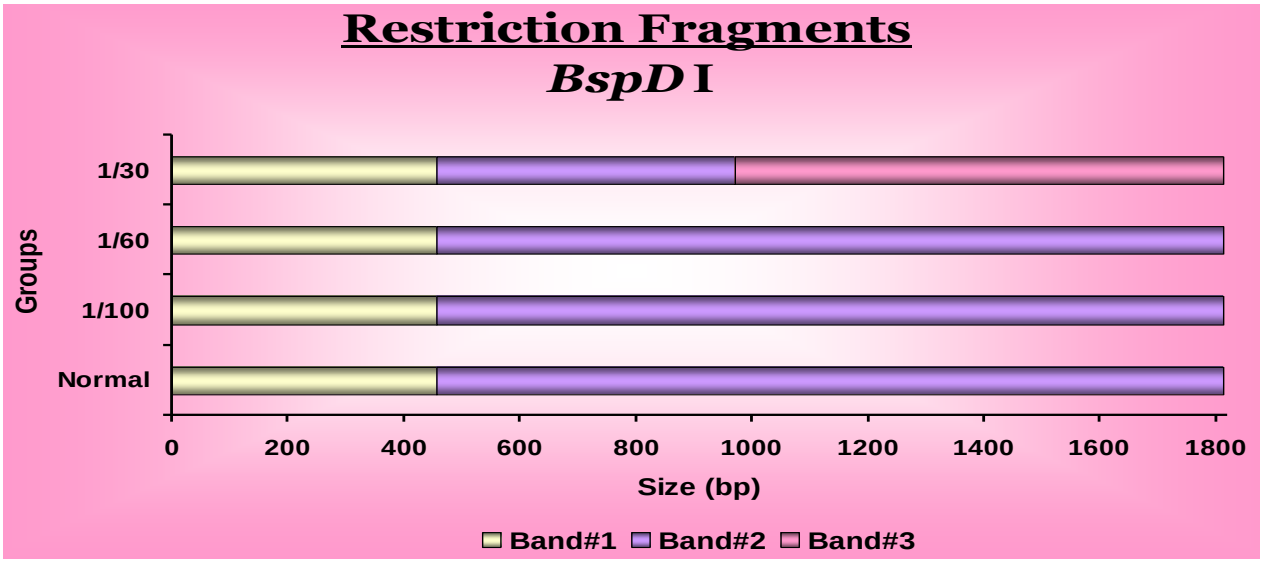

Fig. (17): Shows the fragments lengths of BChE gene predicated from restriction by *BspD*I enzyme in the studied samples. 1/30 group is digested into three fragments while the other groups are digested into two fragments.

Table (6): Shows the patterns lengths of BChE gene which were digested by *Sph*I endonuclease in the studied groups.

Bands / Groups	Band#1	Band#2	Band#3	Band#4
Normal	66	75	1671	----------
1/100	66	75	1671	----------
1/60	66	75	1671	----------
1/30	66	75	410	1261

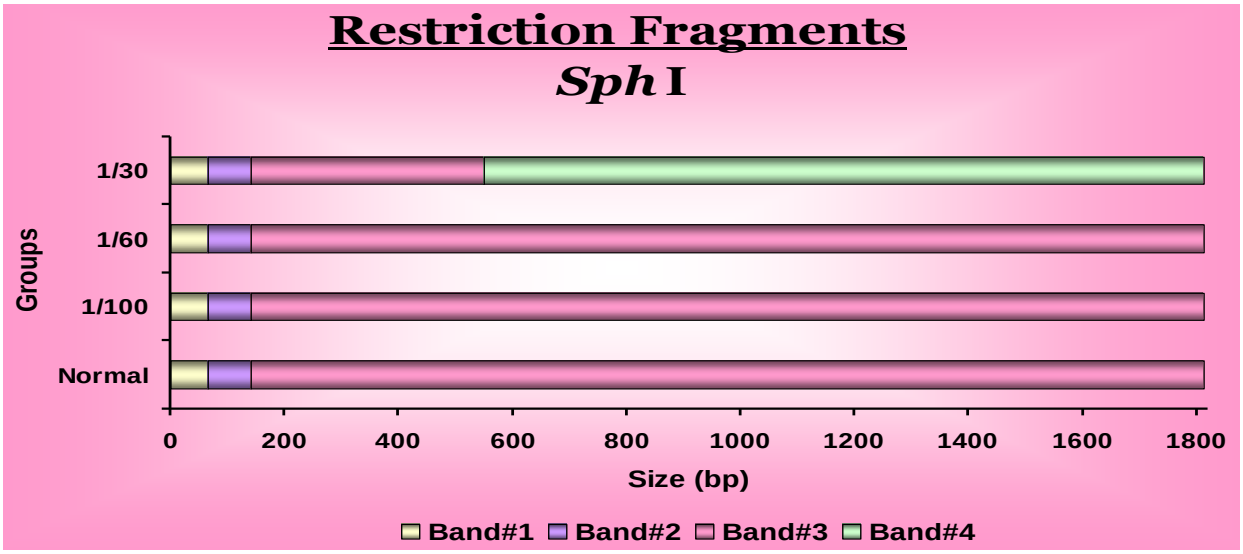

Fig. (18): Represents the patterns lengths of BChE gene which were digested by *Sph*I endonuclease in the studied groups. 1/30 group is digested into four patterns while the other groups are digested into three patterns.

Table (7): Represents the bands lengths of BChE gene which were cut by *Hae*III restriction enzyme in the studied samples.

Bands / Groups	Band#1	Band#2	Band#3	Band#4
Normal	180	243	255	1134
1/100	180	243	255	1134
1/60	180	243	255	1134
1/30	243	435	1134	----------

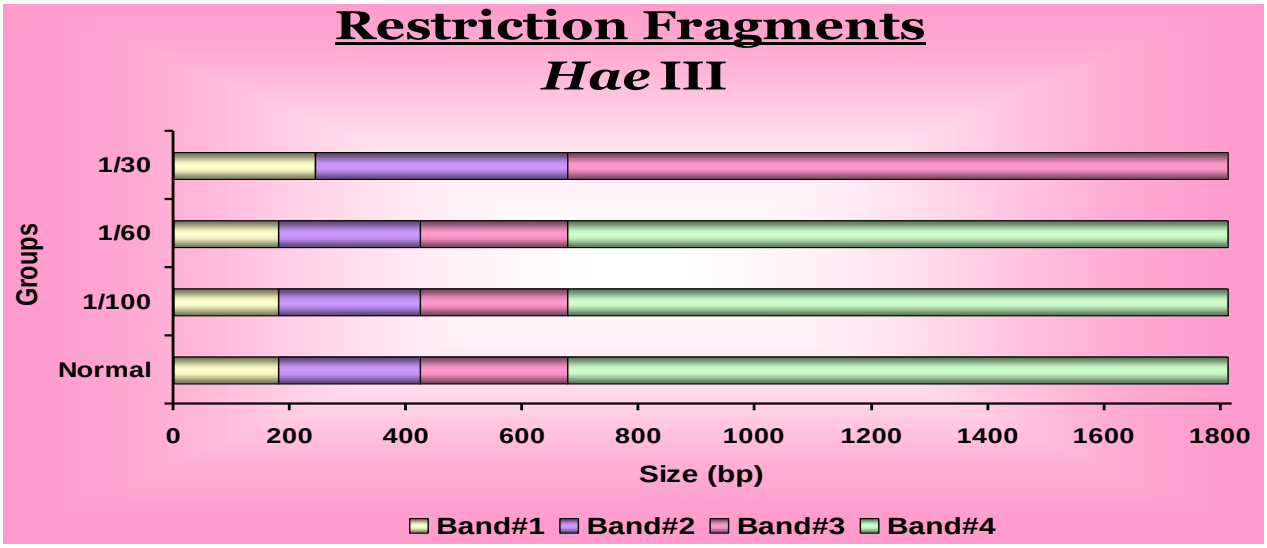

Fig. (19): Shows the bands lengths of BChE gene which were cut by *Hae*III restriction enzyme in the studied samples. 1/30 group is digested into three bands while the other groups are digested into four bands.

Table (8): Represents the patterns lengths of BChE gene produced by digestion with *Mae*II enzyme in the studied groups.

Bands / Groups	Band#1	Band#2	Band#3	Band#4
Normal	607	1205	----------	----------
1/100	607	1205	----------	----------
1/60	285	607	920	----------
1/30	285	607	920	----------

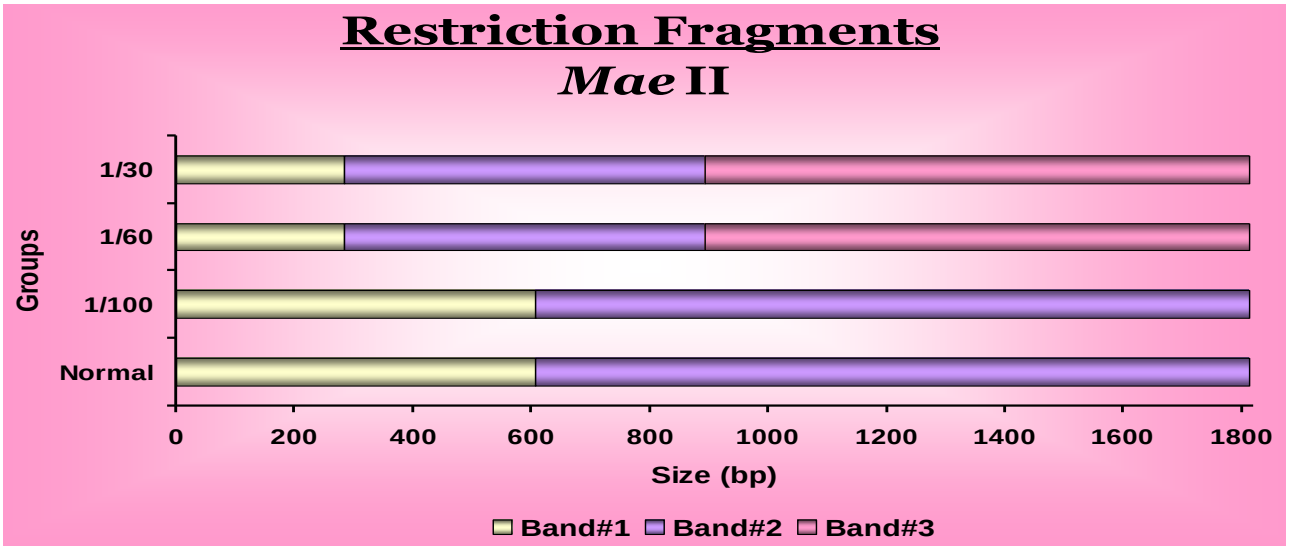

Fig. (20): Shows the patterns lengths of BChE gene produced by digestion with *Mae*II enzyme in the studied groups. 1/30 and 1/60 groups are digested into three patterns while the other groups are digested into two patterns.

Table (9): Represents the patterns lengths of BChE gene which were cut by *Sca*I restriction enzyme in the studied samples.

Bands / Groups	Band#1	Band#2	Band#3	Band#4
Normal	240	1572	----------	----------
1/100	240	389	1183	----------
1/60	240	389	568	615
1/30	240	389	568	615

Fig. (21): Shows the patterns lengths of BChE gene which were cut by *Sca*I restriction enzyme in the studied samples. 1/30 and 1/60 groups are digested into four patterns while 1/100 and normal groups are digested into three and two patterns, respectively.

Table (10): Represents the fragments lengths of BChE gene which were restricted by *Dra*I endonuclease in the studied samples.

Bands / Groups	Band#1	Band#2	Band#3	Band#4
Normal	664	1148	----------	----------
1/100	664	1148	----------	----------
1/60	42	622	1148	----------
1/30	42	233	622	915

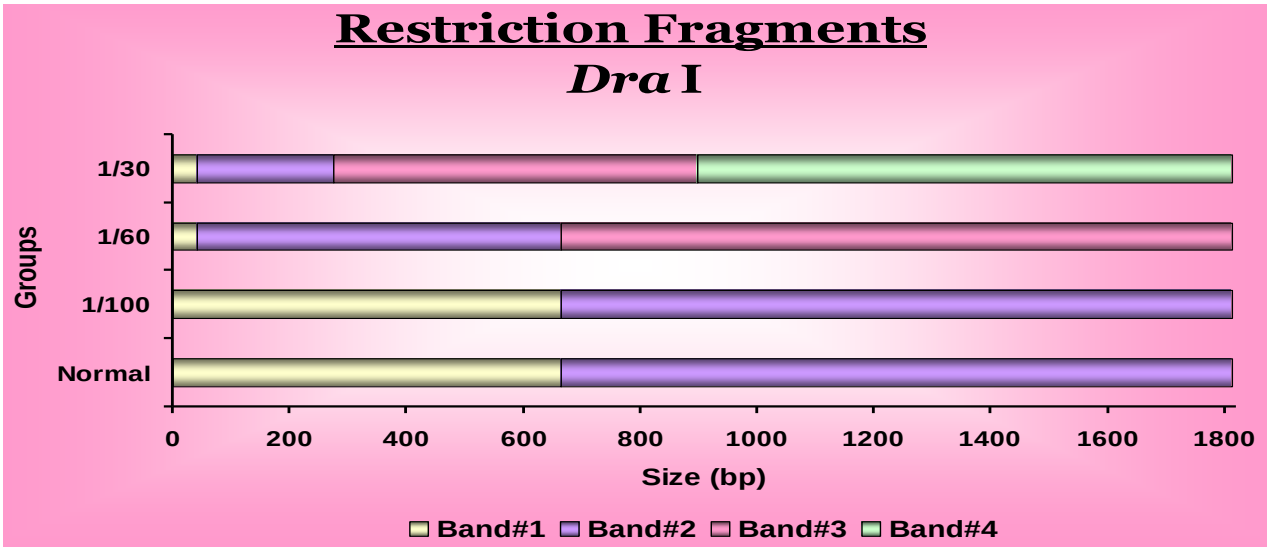

Fig. (22): Shows the fragments lengths of BChE gene which were restricted by *Dra*I endonuclease in the studied samples. 1/30 and 1/60 groups are digested into four and three fragments, respectively while the other groups are digested into two fragments.

Fig. (23): Shows the alignment of BChE nucleotide sequences of normal, 1/100, 1/60 & 1/30 groups. Mutations are set in underlined black color. (.) Refers to one nucleotide mutation in one or three groups, while (:) refers to one nucleotide mutation but in two different groups.

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                10        20        30        40        50        60
           =========+=========+=========+=========+=========+=========+
Normal     ATGCAGACTCAGCATACCAAGGTAACACAGACCCACTTCCTCCTATGGATTCTTCTGCTC
1/100      ATGCAGACTCAGCATACCAAGGTAACACAGACCCACTTCCTCCTATGGATTCTTCTGCTC
1/60       ATGCAGACTCAGCATACCAAGGTAACACAGACCCACTTCCTCCTATGGATTCTTCTGCTC
1/30       ATGCAGACTCAGCATACCAAGGTAACACAGACCCACTTCCTCCTATGGATTCTTCTGCTC
           ############################################################
                70        80        90       100       110       120
           =========+=========+=========+=========+=========+=========+
Normal     TGCATGCCTTTTGGGAAGTCACACACTGAAGAAGACTTCATAATTACAACCAAGACCGGA
1/100      TGCATGCCTTTTGGGAAGTCACACACTGAAGAAGACTTCATAATTACAACCAAGACCGGA
1/60       TGCATGCCTTTTGGGAAGTCACACACTGAAGAAGACTTCATAATTACAACCAAGACCGGA
1/30       TGCATGCCTTTTGGGAAGTCACACACTGAAGAAGACTTCATAATTACAACCAAGACCGGA
           ############################################################
               130       140       150       160       170       180
           =========+=========+=========+=========+=========+=========+
Normal     AGGGTCCGAGGGCTGAGCATGCCAGTTCTTGGTGGCACGGTGACTGCCTTTCTCGGTATC
1/100      AGGGTCCGAGGGCTGAGCATGCCAGTTCTTGGTGGCACGGTGACTGCCTTTCTCGGTATC
1/60       AGGGTCCGAGGGCTGAGCATGCCAGTTCTTGGTGGCACGGTGACTGCCTTTCTCGGTATC
1/30       AGGGTCCGAGGGCTGAGCATGCCAGTTCTTGGTGGCACGGTGACTGCCTTTCTCGGTATC
           ############################################################
               190       200       210       220       230       240
           =========+=========+=========+=========+=========+=========+
Normal     CCCTATGCACAACCTCCTCTGGGTAGCCTAAGATTCAAAAAGCCGCAACCCTTAAACAAA
1/100      CCCTATGCACAACCTCCTCTGGGTAGCCTAAGATTCAAAAAGCCGCAACCCTTAAACAAA
1/60       CCCTATGCACAACCTCCTCTGGGTAGCCTAAGATTCAAAAAGCCGCAACCCTTAAACAAA
1/30       CCCTATGCACAACCTCCTCTGGGTAGCCTAAGATTCAAAAAGCCGCAACCTTTAAACAAA
           ###########################################################.##
               250       260       270       280       290       300
           =========+=========+=========+=========+=========+=========+
Normal     TGGCCTGACATCCATAATGCCACTCAATATGCAAATTCTTGTTATCAGAACATAGACCAA
1/100      TGGCCTGACATCCATAATGCCACTCAATATGCAAATTCTTGTTATCAGAACATAGACCAA
1/60       TGGCCTGACATCCATAATGCCACTCAATATGCAAATTCTTGTTATCAGAACATAGACCAA
1/30       TGGCCTGACATCCATAATGCCACTCAATATGCAAATTCTTGTTATCAGAACATAGACCAA
           ############################################################
               310       320       330       340       350       360
           =========+=========+=========+=========+=========+=========+
Normal     GCCTTCCCAGGCTTCCAGGGGTCAGAAATGTGGAATCCAAACACAAACCTCAGTGAAGAC
1/100      GCCTTCCCAGGCTTCCAGGGGTCAGAAATGTGGAATCCAAACACAAACCTCAGTGAAGAC
1/60       GCCTTCCCAGGCTTCCAGGGGTCAGAAATGTGGAATCCAAACACAAACCTCAGTGAAGAC
1/30       GCCTTCCCAGGCTTCCAGGGGTCAGAAATGTGGAATCCAAACACAAACCTCAGTGAAGAC
           ############################################################
               370       380       390       400       410       420
           =========+=========+=========+=========+=========+=========+
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1/100      TGCTTGTATCTGAATGTTTGGATTCCAGTACTGAAGCCTAAAAATGCCACTGTCATGGTA
1/60       TGCTTGTATCTGAATGTTTGGATTCCAGTACTGAAGCCTAAAAATGCCACTGTCATGGTA
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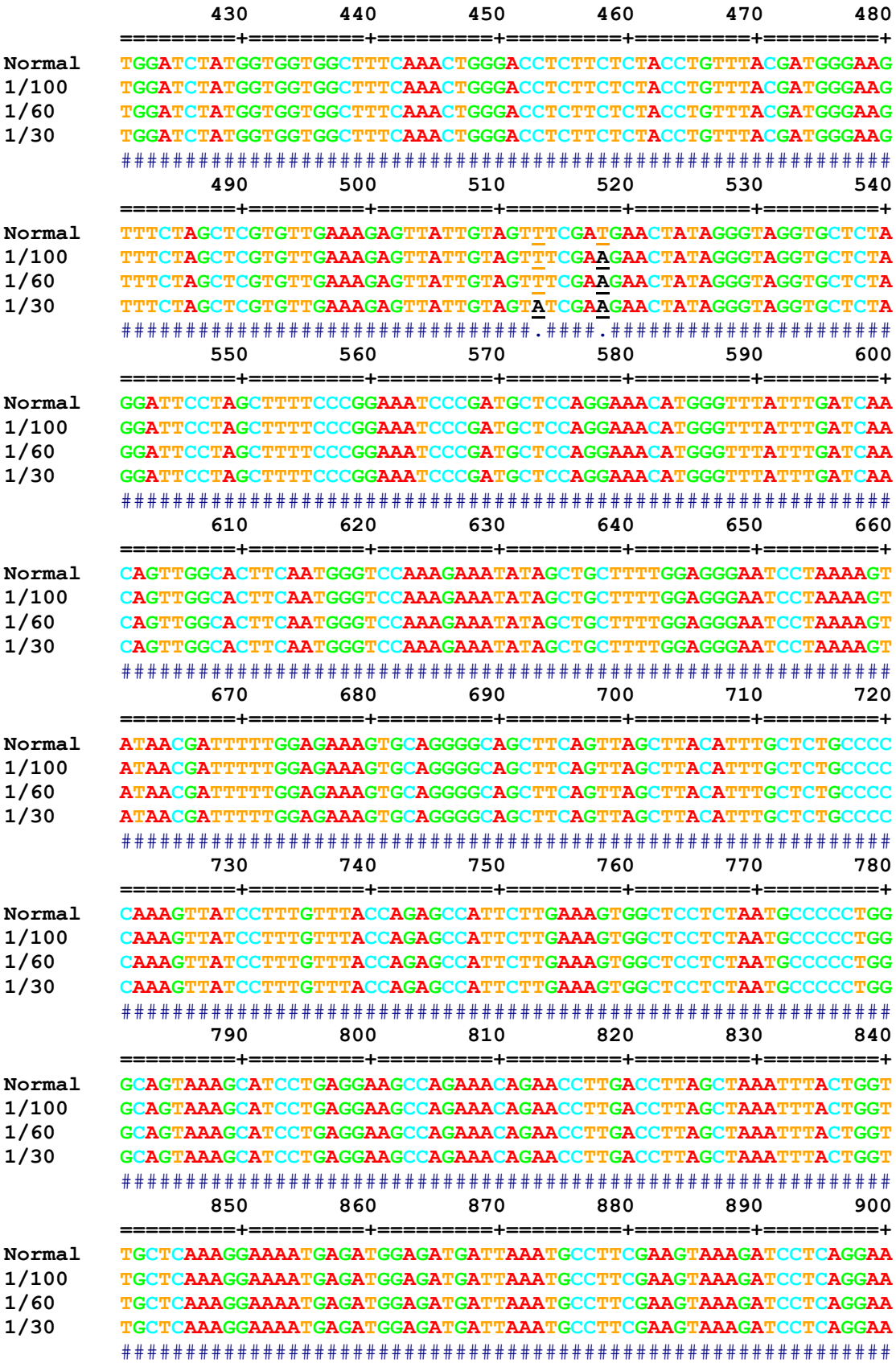
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1/60 TGGATCTATGGTGGTGGCTTTCAAACTGGGACCTCTTCTCTACCTGTTTACGATGGGAAG
1/30 TGGATCTATGGTGGTGGCTTTCAAACTGGGACCTCTTCTCTACCTGTTTACGATGGGAAG
490 500 510 520 530 540
Normal TTTCTAGCTCGTGTTGAAAGAGTTATTGTAGTTTCGATGAACTATAGGGTAGGTGCTCTA
1/100 TTTCTAGCTCGTGTTGAAAGAGTTATTGTAGTTTCGAAGAACTATAGGGTAGGTGCTCTA
1/60 TTTCTAGCTCGTGTTGAAAGAGTTATTGTAGTTTCGAAGAACTATAGGGTAGGTGCTCTA
1/30 TTTCTAGCTCGTGTTGAAAGAGTTATTGTAGTATCGAAGAACTATAGGGTAGGTGCTCTA
550 560 570 580 590 600
Normal GGATTCCTAGCTTTTCCCGGAAATCCCGATGCTCCAGGAAACATGGGTTTATTTGATCAA
1/100 GGATTCCTAGCTTTTCCCGGAAATCCCGATGCTCCAGGAAACATGGGTTTATTTGATCAA
1/60 GGATTCCTAGCTTTTCCCGGAAATCCCGATGCTCCAGGAAACATGGGTTTATTTGATCAA
1/30 GGATTCCTAGCTTTTCCCGGAAATCCCGATGCTCCAGGAAACATGGGTTTATTTGATCAA
610 620 630 640 650 660
Normal CAGTTGGCACTTCAATGGGTCCAAAGAAATATAGCTGCTTTTGGAGGGAATCCTAAAAGT
1/100 CAGTTGGCACTTCAATGGGTCCAAAGAAATATAGCTGCTTTTGGAGGGAATCCTAAAAGT
1/60 CAGTTGGCACTTCAATGGGTCCAAAGAAATATAGCTGCTTTTGGAGGGAATCCTAAAAGT
1/30 CAGTTGGCACTTCAATGGGTCCAAAGAAATATAGCTGCTTTTGGAGGGAATCCTAAAAGT
670 680 690 700 710 720
Normal ATAACGATTTTTGGAGAAAGTGCAGGGGCAGCTTCAGTTAGCTTACATTTGCTCTGCCCC
1/100 ATAACGATTTTTGGAGAAAGTGCAGGGGCAGCTTCAGTTAGCTTACATTTGCTCTGCCCC
1/60 ATAACGATTTTTGGAGAAAGTGCAGGGGCAGCTTCAGTTAGCTTACATTTGCTCTGCCCC
1/30 ATAACGATTTTTGGAGAAAGTGCAGGGGCAGCTTCAGTTAGCTTACATTTGCTCTGCCCC
730 740 750 760 770 780
Normal CAAAGTTATCCTTTGTTTACCAGAGCCATTCTTGAAAGTGGCTCCTCTAATGCCCCCTGG
1/100 CAAAGTTATCCTTTGTTTACCAGAGCCATTCTTGAAAGTGGCTCCTCTAATGCCCCCTGG
1/60 CAAAGTTATCCTTTGTTTACCAGAGCCATTCTTGAAAGTGGCTCCTCTAATGCCCCCTGG
1/30 CAAAGTTATCCTTTGTTTACCAGAGCCATTCTTGAAAGTGGCTCCTCTAATGCCCCCTGG
790 800 810 820 830 840
Normal GCAGTAAAGCATCCTGAGGAAGCCAGAAACAGAACCTTGACCTTAGCTAAATTTACTGGT
1/100 GCAGTAAAGCATCCTGAGGAAGCCAGAAACAGAACCTTGACCTTAGCTAAATTTACTGGT
1/60 GCAGTAAAGCATCCTGAGGAAGCCAGAAACAGAACCTTGACCTTAGCTAAATTTACTGGT
1/30 GCAGTAAAGCATCCTGAGGAAGCCAGAAACAGAACCTTGACCTTAGCTAAATTTACTGGT
850 860 870 880 890 900
Normal TGCTCAAAGGAAAATGAGATGGAGATGATTAAATGCCTTCGAAGTAAAGATCCTCAGGAA
1/100 TGCTCAAAGGAAAATGAGATGGAGATGATTAAATGCCTTCGAAGTAAAGATCCTCAGGAA
1/60 TGCTCAAAGGAAAATGAGATGGAGATGATTAAATGCCTTCGAAGTAAAGATCCTCAGGAA
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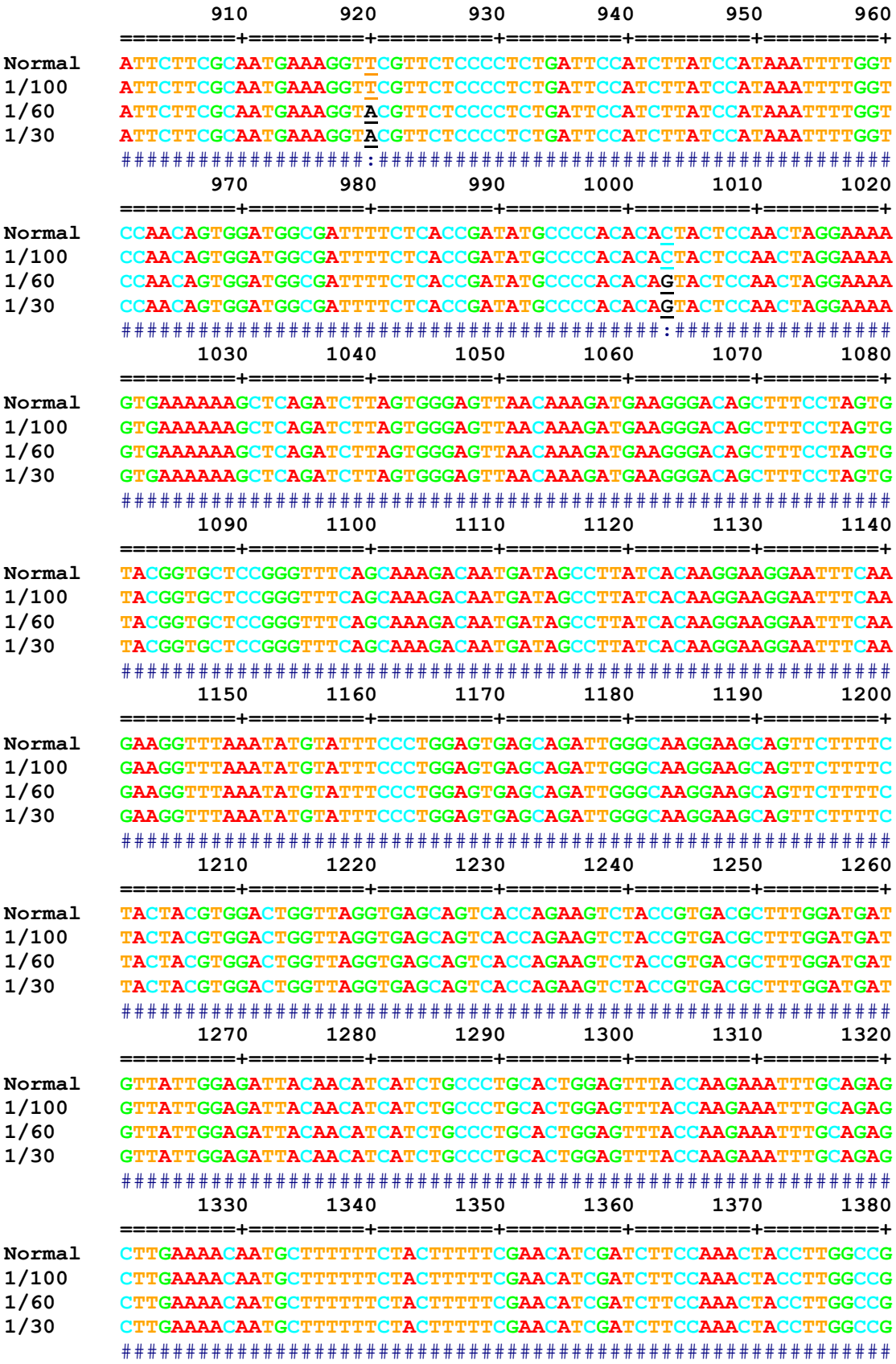
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1/60 ATTCTTCGCAATGAAAGGTACGTTCTCCCCTCTGATTCCATCTTATCCATAAATTTTGGT
1/30 ATTCTTCGCAATGAAAGGTACGTTCTCCCCTCTGATTCCATCTTATCCATAAATTTTGGT
970 980 990 1000 1010 1020
Normal CCAACAGTGGATGGCGATTTTCTCACCGATATGCCCCACACACTACTCCAACTAGGAAAA
1/100 CCAACAGTGGATGGCGATTTTCTCACCGATATGCCCCACACACTACTCCAACTAGGAAAA
1/60 CCAACAGTGGATGGCGATTTTCTCACCGATATGCCCCACACAGTACTCCAACTAGGAAAA
1/30 CCAACAGTGGATGGCGATTTTCTCACCGATATGCCCCACACAGTACTCCAACTAGGAAAA
1030 1040 1050 1060 1070 1080
Normal GTGAAAAAAGCTCAGATCTTAGTGGGAGTTAACAAAGATGAAGGGACAGCTTTCCTAGTG
1/100 GTGAAAAAAGCTCAGATCTTAGTGGGAGTTAACAAAGATGAAGGGACAGCTTTCCTAGTG
1/60 GTGAAAAAAGCTCAGATCTTAGTGGGAGTTAACAAAGATGAAGGGACAGCTTTCCTAGTG
1/30 GTGAAAAAAGCTCAGATCTTAGTGGGAGTTAACAAAGATGAAGGGACAGCTTTCCTAGTG
1090 1100 1110 1120 1130 1140
Normal TACGGTGCTCCGGGTTTCAGCAAAGACAATGATAGCCTTATCACAAGGAAGGAATTTCAA
1/100 TACGGTGCTCCGGGTTTCAGCAAAGACAATGATAGCCTTATCACAAGGAAGGAATTTCAA
1/60 TACGGTGCTCCGGGTTTCAGCAAAGACAATGATAGCCTTATCACAAGGAAGGAATTTCAA
1/30 TACGGTGCTCCGGGTTTCAGCAAAGACAATGATAGCCTTATCACAAGGAAGGAATTTCAA
1150 1160 1170 1180 1190 1200
Normal GAAGGTTTAAATATGTATTTCCCTGGAGTGAGCAGATTGGGCAAGGAAGCAGTTCTTTTC
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1/30 GAAGGTTTAAATATGTATTTCCCTGGAGTGAGCAGATTGGGCAAGGAAGCAGTTCTTTTC
1210 1220 1230 1240 1250 1260
Normal TACTACGTGGACTGGTTAGGTGAGCAGTCACCAGAAGTCTACCGTGACGCTTTGGATGAT
1/100 TACTACGTGGACTGGTTAGGTGAGCAGTCACCAGAAGTCTACCGTGACGCTTTGGATGAT
1/60 TACTACGTGGACTGGTTAGGTGAGCAGTCACCAGAAGTCTACCGTGACGCTTTGGATGAT
1/30 TACTACGTGGACTGGTTAGGTGAGCAGTCACCAGAAGTCTACCGTGACGCTTTGGATGAT
1270 1280 1290 1300 1310 1320
Normal GTTATTGGAGATTACAACATCATCTGCCCTGCACTGGAGTTTACCAAGAAATTTGCAGAG
1/100 GTTATTGGAGATTACAACATCATCTGCCCTGCACTGGAGTTTACCAAGAAATTTGCAGAG
1/60 GTTATTGGAGATTACAACATCATCTGCCCTGCACTGGAGTTTACCAAGAAATTTGCAGAG
1/30 GTTATTGGAGATTACAACATCATCTGCCCTGCACTGGAGTTTACCAAGAAATTTGCAGAG
1330 1340 1350 1360 1370 1380
Normal CTTGAAAACAATGCTTTTTTCTACTTTTTCGAACATCGATCTTCCAAACTACCTTGGCCG
1/100 CTTGAAAACAATGCTTTTTTCTACTTTTTCGAACATCGATCTTCCAAACTACCTTGGCCG
1/60 CTTGAAAACAATGCTTTTTTCTACTTTTTCGAACATCGATCTTCCAAACTACCTTGGCCG
1/30 CTTGAAAACAATGCTTTTTTCTACTTTTTCGAACATCGATCTTCCAAACTACCTTGGCCG

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                1390       1400       1410       1420       1430       1440
              ==========+==========+==========+==========+==========+==========+
Normal        GAATGGATGGGAGTGATGCATGGCTATGAAATTGAATTTGTGTTTGGCTTACCTCTGGGA
1/100         GAATGGATGGGAGTGATGCATGGCTATGAAATTGAATTTGTGTTTGGCTTACCTCTGGGA
1/60          GAATGGATGGGAGTGATGCATGGCTATGAAATTGAATTTGTGTTTGGCTTACCTCTGGGA
1/30          GAATGGATGGGAGTGATGCATGCCTATGAAATTGAATTTGTGTTTGGCTTACCTCTGGGA
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                1450       1460       1470       1480       1490       1500
              ==========+==========+==========+==========+==========+==========+
Normal        AGAAGAGTTAATTATACGAGAGCTGAGGAAATCTTTAGTCGATCCATAATGAAAACTTGG
1/100         AGAAGAGTTAATTATACGAGAGCTGAGGAAATCTTTAGTCGATCCATAATGAAAACTTGG
1/60          AGAAGAGTTAATTATACGAGAGCTGAGGAAATCTTTAGTCGATCCATAATGAAAACTTGG
1/30          AGAAGAGTTAATTATACGAGAGCTGAGGAAATCTTTAGTCGATCCATAATGAAAACTTGG
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                1510       1520       1530       1540       1550       1560
              ==========+==========+==========+==========+==========+==========+
Normal        GCAAATTTTGCAAAATATGGACATCCCAATGGGACCCAGGGCAATAGCACAATGTGGCCT
1/100         GCAAATTTTGCAAAATATGGACATCCCAATGGGACCCAGGGCAATAGCACAATGTGGCCT
1/60          GCAAATTTTGCAAAATATGGACATCCCAATGGGACCCAGGGCAATAGCACAATGTGGCCT
1/30          GCAAATTTTGCAAAATATGGACATCCCAATGGGACCCAGGGCAATAGCACAATGTNNNNT
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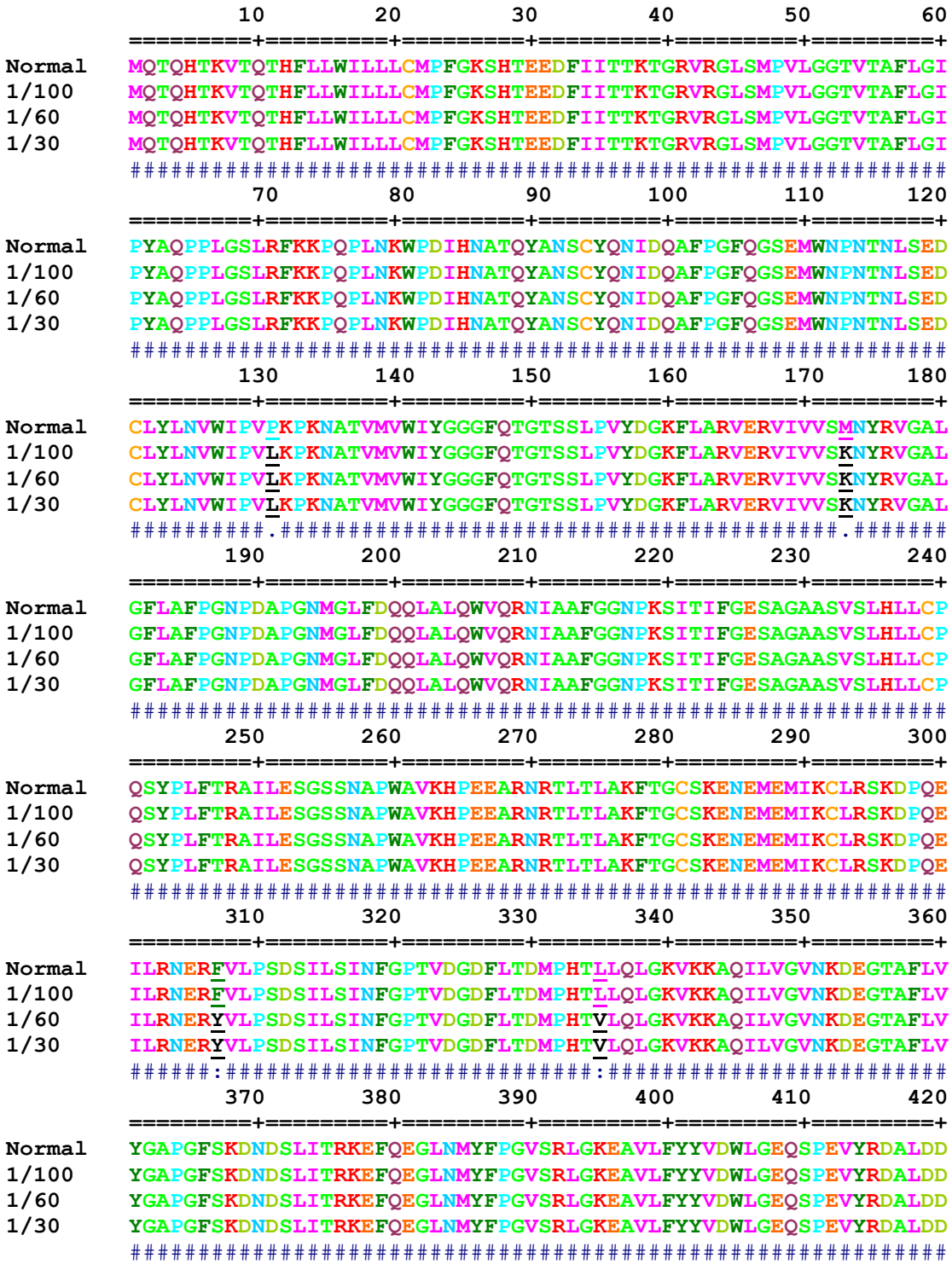
                1570       1580       1590       1600       1610       1620
              ==========+==========+==========+==========+==========+==========+
Normal        GTCTTCACAAGTACTGAACAAAAATACCTAACATTGAACACAGAGAAGTCAAAAATATAC
1/100         GTCTTCACAAGTACTGAACAAAAATACCTAACATTGAACACAGAGAAGTCAAAAATATAC
1/60          GTCTTCACAAGTACTGAACAAAAATACCTAACATTGAACACAGAGAAGTCAAAAATATAC
1/30          GTCTTCACAAGTACTGAACAAAAATACCTAACATTGAACACAGAGAAGTCAAAAATATAC
              ############################################################
                1630       1640       1650       1660       1670       1680
              ==========+==========+==========+==========+==========+==========+
Normal        TCTAAACTTCGTGCTCCCCAATGTCAGTTCTGGAGACTATTTTTTCCAAAAGTCTTGGAA
1/100         TCTAAACTTCGTGCTCCCCAATGTCAGTTCTGGAGACTATTTTTTCCAAAAGTCTTGGAA
1/60          TCTAAACTTCGTGCTCCCCAATGTCAGTTCTGGAGACTATTTTTTCCAAAAGTCTTGGAA
1/30          TCTAAACTTCGTGCTCCCCAATGTCAGTTCTGGAGACTATTTTTTCCAAAAGTCTTGGAA
              ############################################################
                1690       1700       1710       1720       1730       1740
              ==========+==========+==========+==========+==========+==========+
Normal        ATGACAGGAGATATTGATGAAACGGAGCAAGAGTGGAAGGCAGGATTTCATCGCTGGAGC
1/100         ATGACAGGAGATATTGATGAAACGGAGCAAGAGTGGAAGGCAGGATTTCATCGCTGGAGC
1/60          ATGACAGGAGATATTGATGAAACGGAGCAAGAGTGGAAGGCAGGATTTCATCGCTGGAGC
1/30          ATGACAGGAGATATTGATGAAACGGAGCAAGAGTGGAAGGCAGGATTTCATCGCTGGAGC
              ############################################################
                1750       1760       1770       1780       1790       1800
              ==========+==========+==========+==========+==========+==========+
Normal        AATTACATGATGGACTGGCAAAATCAATTTAACGATTACACTAGCAAGAAAGAGAGCTGT
1/100         AATTACATGATGGACTGGCAAAATCAATTTAACGATTACACTAGCAAGAAAGAGAGCTGT
1/60          AATTACATGATGGACTGGCAAAATCAATTTAAAGATTACACTAGCAAGAAAGAGAGCTGT
1/30          AATTACATGATGGACTGGCAAAATCAATTTAAAGATTACACTAGCAAGAAAGAGAGCTGT
              ##################################:#########################
                1810
              ==========+==
Normal        ACAGCTCTCTAA
1/100         ACAGCTCTCTAA
1/60          ACAGCTCTCTAA
1/30          ACAGCTCTCTAA
              ############
```

Fig. (24): Shows the alignment of BChE protein sequences of normal, 1/100, 1/60 & 1/30 groups. Amino acid changes are set in underlined black color. (.) Refers to one amino acid change in one or three groups, while (:) refers to one amino acid change but in two different groups.



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```
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Normal         VIGDYNIICPALEFTKKFAELENNAFFYFFEHRSSKLPWPEWMGVMHGYEIEFVFGLPLG
1/100          VIGDYNIICPALEFTKKFAELENNAFFYFFEHRSSKLPWPEWMGVMHGYEIEFVFGLPLG
1/60           VIGDYNIICPALEFTKKFAELENNAFFYFFEHRSSKLPWPEWMGVMHGYEIEFVFGLPLG
1/30           VIGDYNIICPALEFTKKFAELENNAFFYFFEHRSSKLPWPEWMGVMHAYEIEFVFGLPLG
               ##################################################.############
                 490        500        510        520        530        540
               ---------+---------+---------+---------+---------+---------+
Normal         RRVNYTRAEEIFSRSIMKTWANFAKYGHPNGTQGNSTMWPVFTSTEQKYLTLNTEKSKIY
1/100          RRVNYTRAEEIFSRSIMKTWANFAKYGHPNGTQGNSTMWPVFTSTEQKYLTLNTEKSKIY
1/60           RRVNYTRAEEIFSRSIMKTWANFAKYGHPNGTQGNSTMWPVFTSTEQKYLTLNTEKSKIY
1/30           RRVNYTRAEEIFSRSIMKTWANFAKYGHPNGTQGNSTM??VFTSTEQKYLTLNTEKSKIY
               ########################################..####################
                 550        560        570        580        590        600
               ---------+---------+---------+---------+---------+---------+
Normal         SKLRAPQCQFWRLFFPKVLEMTGDIDETEQEWKAGFHRWSNYMMDWQNQFNDYTSKKESC
1/100          SKLRAPQCQFWRLFFPKVLEMTGDIDETEQEWKAGFHRWSNYMMDWQNQFNDYTSKKESC
1/60           SKLRAPQCQFWRLFFPKVLEMTGDIDETEQEWKAGFHRWSNYMMDWQNQFKDYTSKKESC
1/30           SKLRAPQCQFWRLFFPKVLEMTGDIDETEQEWKAGFHRWSNYMMDWQNQFKDYTSKKESC
               ##########################################################:#########
               ===
Normal         TAL
1/100          TAL
1/60           TAL
1/30           TAL
               ###
```

Table (11): Represents the different changes in nucleotides, amino acids and their positions after BChE gene digestion by different endonucleases in the studied groups.

DNA Molecule / Treated Groups	Nucleotide Change	Nucleotide Change Position	Amino Acid Change	Amino Acid Change Position	Restriction Enzymes Used
1/100	C►T	392	P►L	131	*Sca*I
	T►A	518	M►K	173	*Bst*BI
1/60	C►T	392	P►L	131	*Sca*I
	T►A	518	M►K	173	*Bst*BI
	T►A	920	F►Y	307	*Mae*II
	C►G	1003	L►V	335	*Sca*I
	C►A	1773	N►K	591	*Dra*I
1/30	C►T	231	No change	No change	*Dra*I
	C►T	392	P►L	131	*Sca*I
	T►A	513	No change	No change	*Bsp*DI
	T►A	518	M►K	173	*Bst*BI
	T►A	920	F►Y	307	*Mae*II
	C►G	1003	L►V	335	*Sca*I
	G►C	1403	G►A	468	*Sph*I
	GGCC►N	1556 or 1557 or 1558 or 1559	W►? or P►?	519 or 520	*Hae*III
	C►A	1773	N►K	591	*Dra*I

Table (14): Represents the Distance Matrix of the four studied groups (normal, 1/100, 1/60 & 1/30).

Distance Matrix (Substitutions per site)				
Groups	**Normal**	**1/100**	**1/60**	**1/30**
Normal	----------	0.0011049	0.0027650	0.0044396
1/100	0.0011049	----------	0.0016577	0.0033266
1/60	0.0027650	0.0016577	----------	0.0016615
1/30	0.0044396	0.0033266	0.0016615	----------

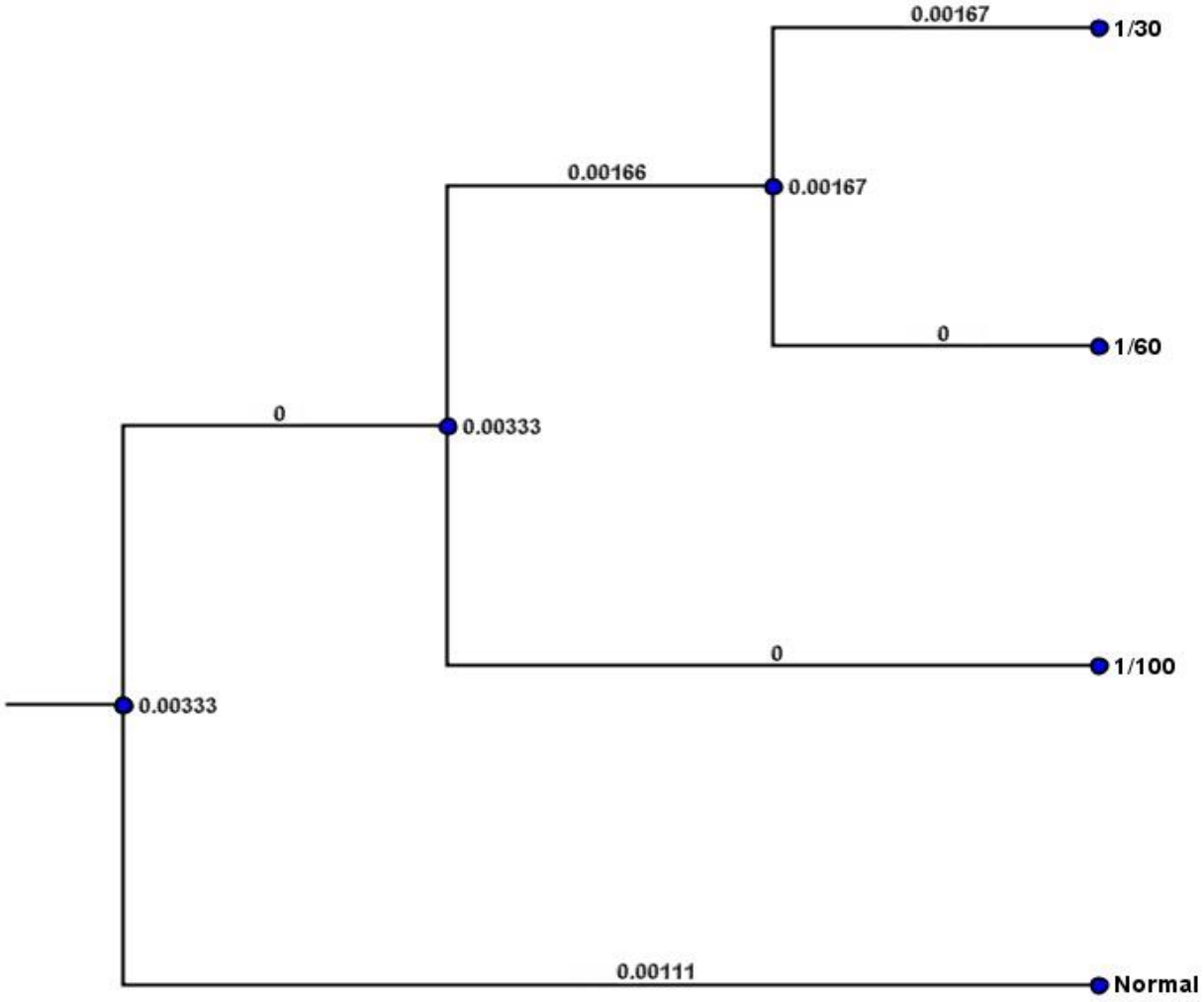

Fig. (25): Shows rooted Phylogenetic distance tree (cladogram) of the four studied groups. These groups are divided into two clusters, (normal &1/100) and (1/60 & 1/30).

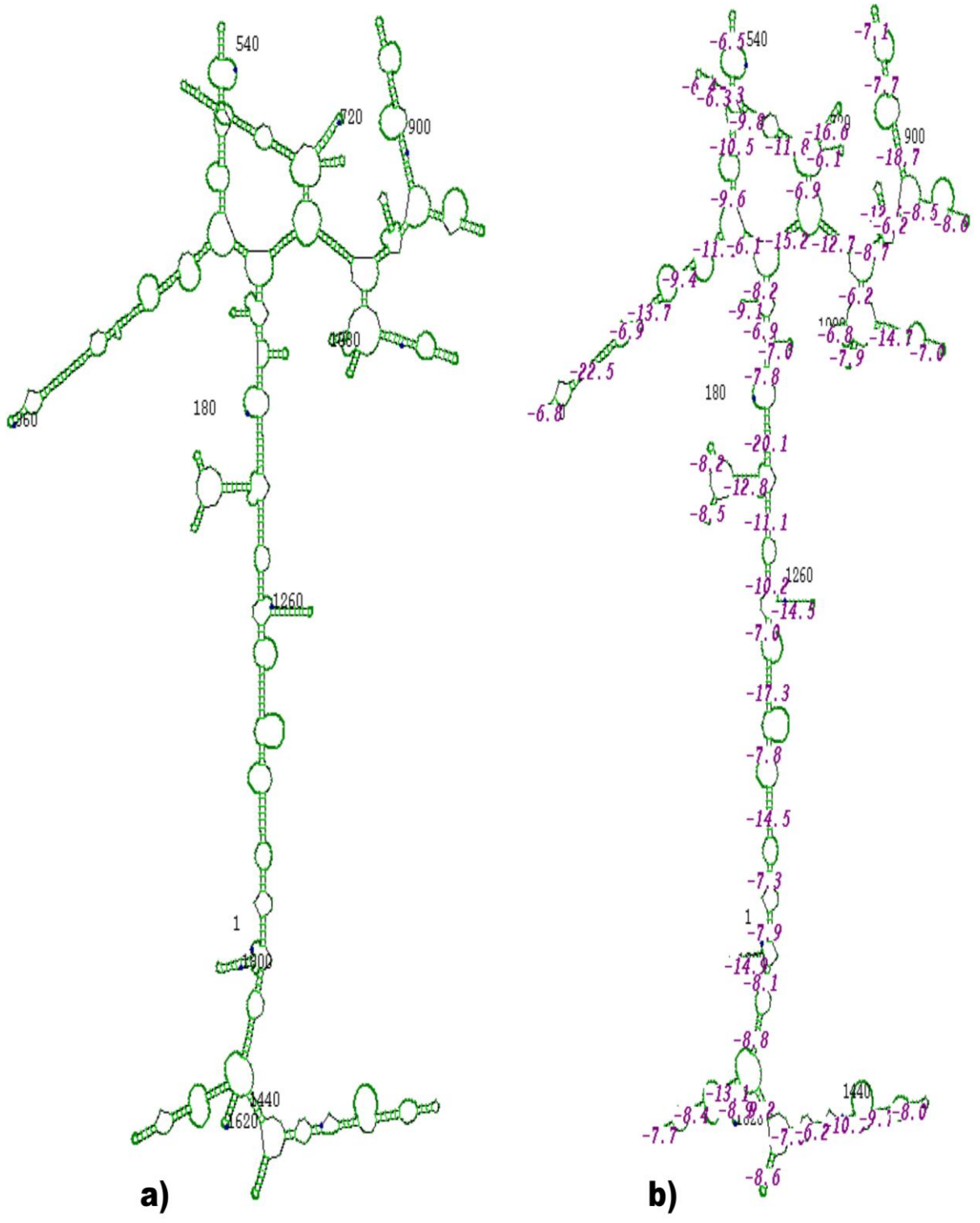

Fig. (26): Shows RNA secondary structure prediction of normal BChE nucleotide sequence. (a) RNA secondary structure with nucleotide number positions. (b) RNA secondary structure with nucleotide number positions & stems free energies. The structure free energy is -338.7 kcal /mol.

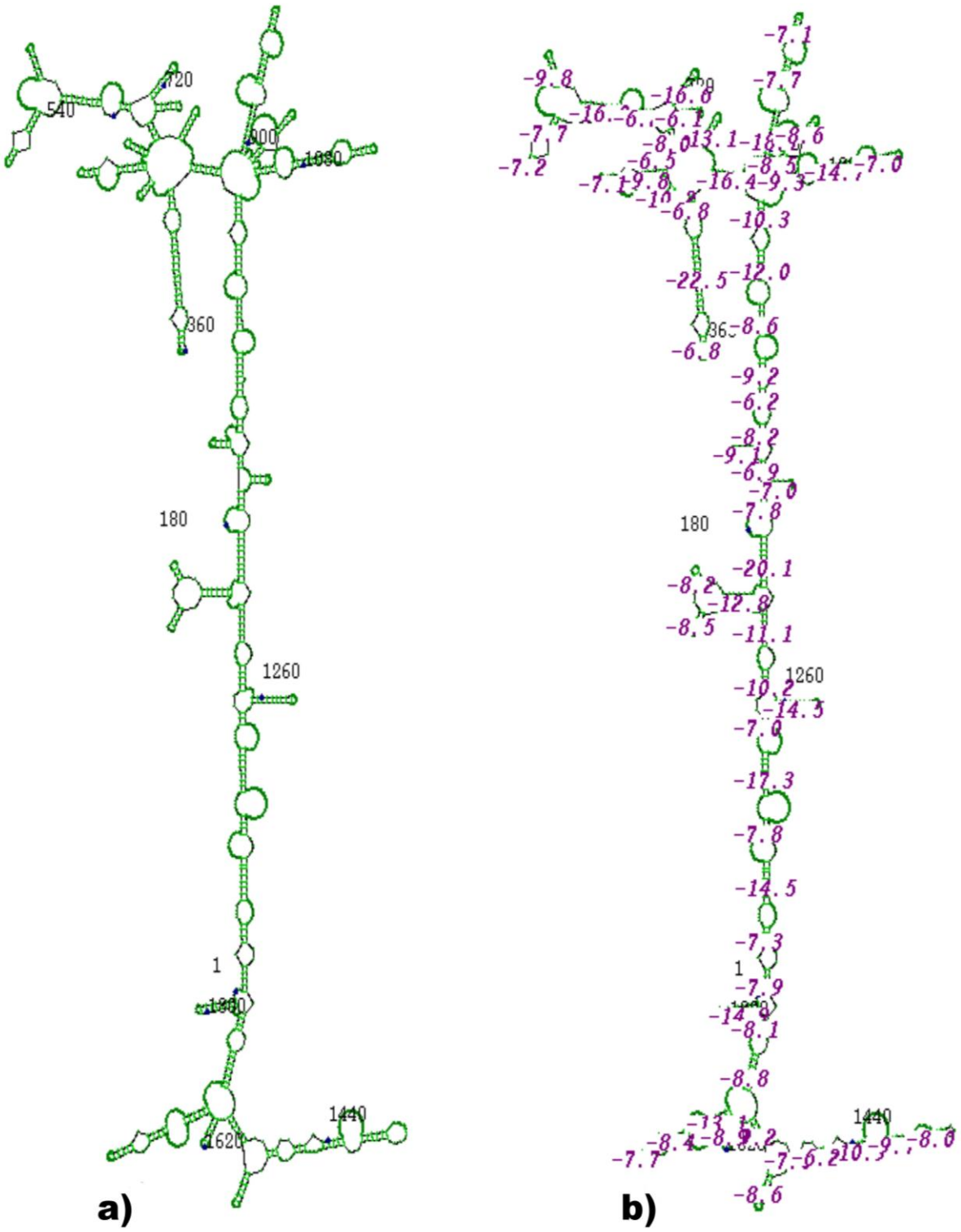

Fig. (27): Shows RNA secondary structure prediction of 1/100 BChE nucleotide sequence. (a) RNA secondary structure with nucleotide number positions. (b) RNA secondary structure with nucleotide number positions & stems free energies. The structure free energy is -338.6 kcal /mol.

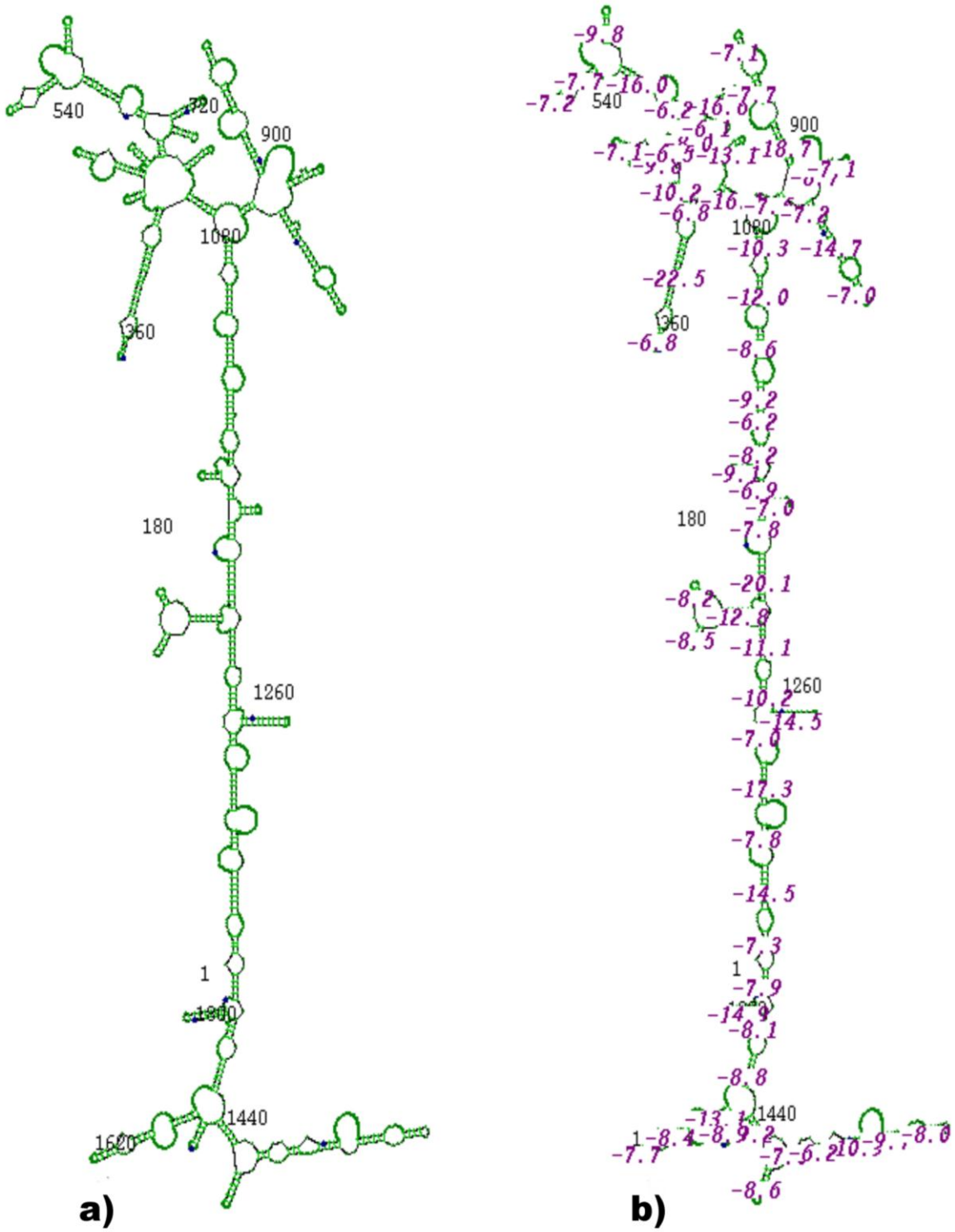

Fig. (28): Shows RNA secondary structure prediction of 1/60 BChE nucleotide sequence. (a) RNA secondary structure with nucleotide number positions. (b) RNA secondary structure with nucleotide number positions & stems free energies. The structure free energy is -342.8 kcal /mol.

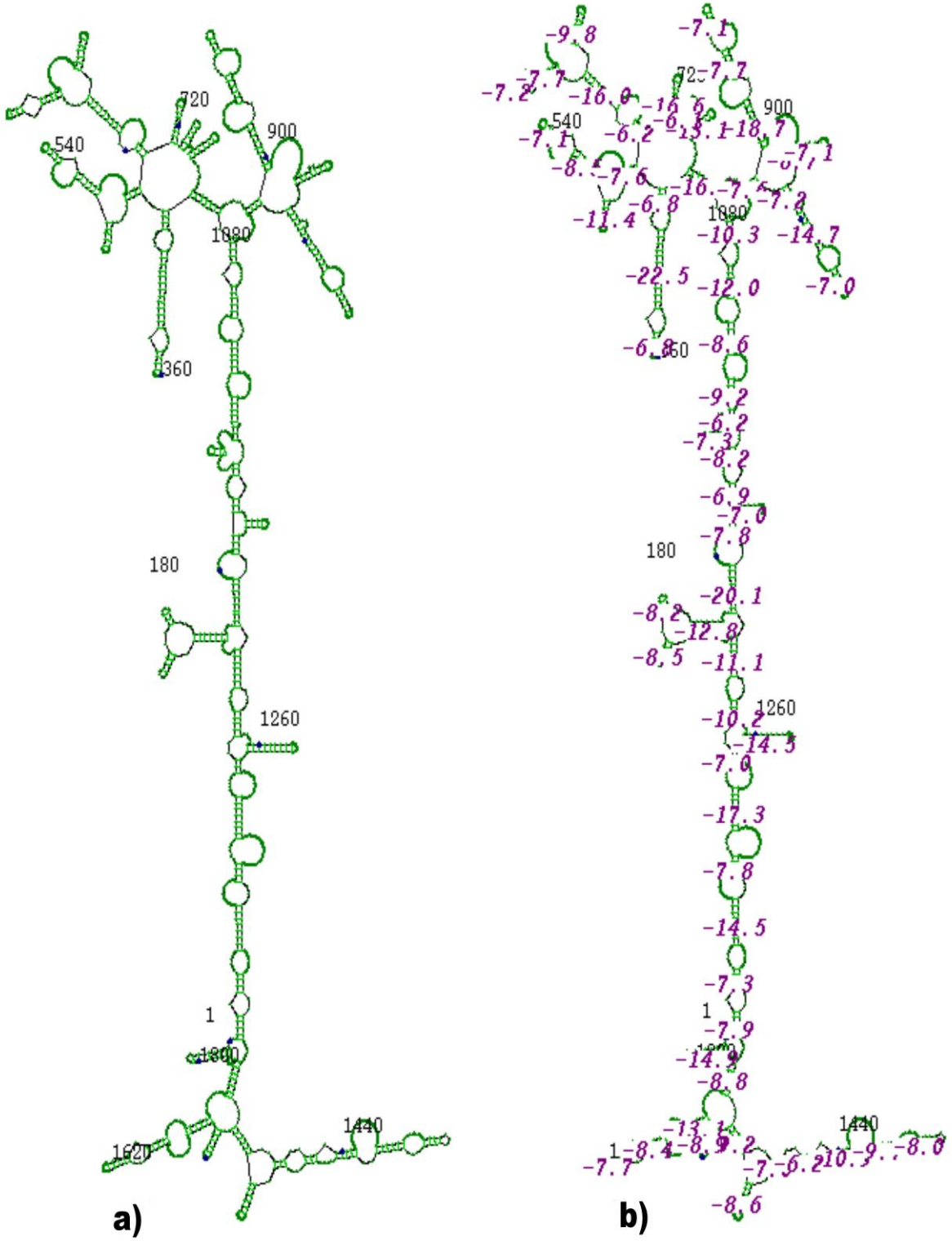

Fig. (29): Shows RNA secondary structure prediction of 1/30 BChE nucleotide sequence. (a) RNA secondary structure with nucleotide number positions. (b) RNA secondary structure with nucleotide number positions & stems free energies. The structure free energy is -325.8 kcal /mol.