Sequence analysis of four caprine mitochondria DNA lineages

Yan-Ping Wu,1,2 Jun-Hong Huo,2 Wei-Jun Guan,1 Jin-Fang Xie,1 Yue-Hui Ma1
1Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China
2Institute of Animal Science, Jiangxi Academy of Agricultural Sciences, Nanchang, China

Abstract

The complete mitochondrial DNA (mtDNA) (16640bp in length) was sequenced from four Chinese goat lineages representing the four major mtDNA haplogroups in goats. A total of 124 single nucleotide polymorphisms (SNPs) were found in encoding regions, and the overall ratio of transitions:transversions was 40:1 revealing a heavy transition/transversion rate in domestic goats. Eighteen non-synonymous sites were found for the total number of SNPs; the sites did not affect the predicted functions of protein for these four goat mtDNA lineages. In the region for coding tRNA and rRNA, SNPs occurred in loops, unstructured single strand and stems that were conformed with the principle of G-U pairing. We came to the conclusion that these substitutions could not change secondary structure of RNAs, and there was no positive selection on goat mitochondrial coding region according to the result of dN/dS (0.0399-0.1529) by comparing the goat with other reported mitochondrial genomes.

Introduction

Genetic analysis of mitochondrial (mt) DNA has been an important tool in understanding goat evolution. Attributes such as high copy number per cell, lack of recombination (Olivo et al., 1983), high substitution rate (Brown et al., 1985) and uniparental inheritance (Giles et al., 1980; Gyllensten et al., 1985) have made mitochondrial DNA (mtDNA) a source of information for phylogenetic studies. The circular mitochondrial genome of goat encodes 13 polypeptides, 22 transfer RNA (tRNA) genes and 2 ribosomal RNA (rRNA) genes necessary for transcription and translation of the mitochondrial genome. Among these genes, only NADH dehydrogenase subunit 6 and eight tRNAs are encoded in the L-strand while the others are encoded by the H-strand. With the exception of the ND2, ND3 and ND5 genes, all peptide-coding genes of the goat mtDNA have a methionine start codon (Parma et al., 2003). Comprehensive studies have demonstrated that goat mtDNA is geographically structured and may be classified into four mtDNA haplogroups observed in China (Chen et al., 2005). Analyses of the previous studies focused on the d-loop that occupies less than 7% of the mtDNA genome (Chen et al., 2005; Joshi et al., 2004; Luikart et al., 2001; Mannen et al., 2001; Naderi et al., 2007) and is a major non-coding region regulating replication and transcription of mtDNAs (Clayton, 1992). Therefore, analysis of the whole mtDNA sequences from various goat lineages would more realistically characterise the evolution of diverse goat lineages. Mitochondrial genome sequences have been widely used in phylogenetic relationship analyses. Currently, the complete mitochondrial genome DNA of human, cattle, pig, horse, donkey, chicken, sheep, goat and yak have been sequenced (Anderson et al., 1981, 1982; Gu et al., 2006; Hiendleder et al., 1998; Lin et al., 1999; Nishihori et al., 2003; Parma et al., 2003; Xu and Arnason, 1994; Xu et al., 1996). Non-synonymous-synonymous substitution rate ratio (dN/dS) is important to evaluate selective pressure based on the protein-coding sequences. When dN/dS is over 1, positive Darwinian selection is inferred; when dN/dS is less than 1, purifying selection is inferred and when dN/dS is less than 1, purifying selection is inferred. Regions where dN/dS is over 1 are potential sites of positive selection.

In this study, the complete mitochondrial DNA (mtDNA) sequences of four goat lineages were sequenced. Characteristics of the goat mitochondrial genome were analysed and the structure of mitochondrial RNA and protein were predicted. Comparisons of the mitochondrial sequence of goat with that of other reported species were also made to analyse the molecular evolution of the goat mitochondrial DNA.

Materials and methods

Sampling and DNA extraction

Goat ear tissues were collected from small remote villages in five provinces or regions of China. Samples of the goat ear tissues were stored at -70°C before DNA extraction. Total genomic DNA was extracted from ear tissue by a standard phenol-chloroform extraction method (Sambrook and Russell, 2000). Details of the breeds, regions and sample sizes are given in Table 1.

DNA amplification and sequencing

Based on the first hypervariable region of mitochondrial DNA study, 4 samples (one per lineage) were collected and sequenced according to the MJ network of 145 domestic goats (Wu et al., 2009). Fifteen pairs of primers (Appendix Table 1) overlapping in mitochondrial DNA were designed with Premier primer 5.0 and the known goat mtDNA sequence (Parma et al., 2003). Polymerase chain reaction (PCR) amplifications were carried out in 25 μL reaction mixtures including each primer (1 μL of a 10 μM solution), dNTPs (1 μL of a 2.5 mM solution), 2.5 μL of 10 x buffer and 0.25 μL of 5 U/μL Taq DNA polymerase (Tiangen). The PCR programme consisted of an initial denaturing step at 95°C for 5 min, 33 amplifi-
cation cycles (94°C for 30 s, 58.5°C for 30 s and 72°C for 30 s) and a final extension at 72°C for 10 min in a PTC200® Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA). The PCR products were sequenced by Shanghai Bioasia Co. (Shanghai, China). The complete sequences of goat mtDNA lineages were deposited in the GenBank database (Accession n. GU229278-GU229281). Hassanin et al. reveal the presence of nusts and multiple sequencing errors, and suggested that the sequences were probably contaminated by a nust fragment of 1881 bp based on a pair of primers located around 12260-13280 bp of the complete goat mtDNA sequences. We carried out further repeated sequencing using a number of short segment primers and the problem was avoided.

Analysis of sequence data

Overlapping contigs were compiled using DNastar Seqman software (DNASTAR Inc., Madison, WI, USA) to assemble a continuous sequence. Sequence alignments with mitochondrial DNA sequence by Bioedit package (Hall, 1999) were used to annotate goat mitochondrial genes. The influence of amino acid sequence mutation on the structure and function of protein was analysed using online alignment of amino acid sequence. The structure of mitochondrial RNA was predicted using RNAstructure version 4.5 and ViennaRNA version 1.5 software. The neighbour-joining (NJ) tree was constructed using the Mega 3.0 software programme (Kumar et al., 2004), with a Kimura 2-parameter model and a bootstrap (1000 replications) test. dN/dS(ω) values were calculated using PAML according to Yang (1997).

Results

Characteristics of four goat mtDNA lineages

We constructed the complete nucleotide sequence of four goat mtDNA lineages. The goat mtDNA was 16640-16642bp in length, the average contents of the base A,T,C and G were 29.5%, 25.8%, 31.9% and 12% in the goat mtDNA encoding region, respectively. Average AT contents in the first, second and third codon positions of the protein genes were 55%, 62.1% and 66.1%, respectively, and the third codon position showed obvious AT bias. In total, 124 SNPs were found among the encoding regions of four lineages, 104 of which were distributed in protein-coding sequences. The overall ratio of transitions:transversions (40:1) revealed a heavy transition bias in domestic goats. The d-loop region variations among four lineages have been reported in previous studies (Wu et al., 2009).

Amino acid sequence analysis

Thirteen protein-coding genes with initial codon of methionine (10 ATG and 3 ATA) were found in the goat mtDNA, among which three genes appeared to end in an incomplete stop codon of TNN, where NN was the 5 terminus of the adjacent tRNA gene, which presumably formed a stop codon by post-transcriptional polyadenylation (Anderson et al., 1981). This observation was consistent with characteris-

RNA secondary structure analysis

Twenty-two tRNA genes and 2 rRNA (12S and 16S rRNA) were found in the goat mtDNA (Parma et al., 2003). The tRNA genes were dispersed along the whole mtDNA genome, RNA secondary structure was more conserved than the primary structure and it contained information concerning the phylogenetic relationship. In this study, 22 tRNA and 2 rRNA sequences of four goat mtDNA lineages were aligned by the mega 3 software package. The result revealed variations of sequences in the 6 tRNA and 2 rRNA. The inferred secondary structures of the 6 tRNA genes showed that the variation was A/G transition. Among 12S rRNA and 16S rRNA of four goat mtDNA lineages, there were 4 and 9 differences, respectively. In the region for coding tRNA and rRNA, SNP occurred in loops, unstructured single strand, and stems that conformed with the principle of G-U pairing (Table 3). It can, therefore, be con-

| Lineage | Breed | Sample size | Resource |
|---------|-------|-------------|----------|
| A       | Langkazi | 1           | Tibet   |
| B       | Yunling Black | 1          | Yunnan  |
| C       | Wuzhumuqin White Cashmere | 1   | Neimeng |
| D       | Tsaidam Cashmere | 1  | Qinghai |

| Gene | Position | SNP of 4 lineage | Amino acid substitutions | Results of polyphen | PSIC score |
|------|----------|------------------|-------------------------|---------------------|------------|
| ND1  | 2943     | T T C T          | lle - Thr               | Benign              | 0.089      |
| COX1 | 6573     | A A A G          | lle - Val               | Benign              | 0.233      |
| COX2 | 7051     | A G G A          | Thr - Ala               | Benign              | 0.055      |
| COX3 | 1302     | A G G A          | Ser - Asx               | Benign              | 0.180      |
| COX4 | 8751     | C T C C          | Ala - Val               | Benign              | 0.400      |
| ND5  | 9060     | T T C T          | Met - Thr               | Benign              | 0.807      |
| ND6  | 9546     | T T C T          | Val - Ala               | Benign              | 0.276      |
| ND4L | 10000    | A A G G          | lle - Val               | Benign              | 0.619      |
| ND3  | 11807    | T T T C          | Met - Thr               | Benign              | 0.591      |
| ND4L | 11870    | G A G G          | Cys - Tyr               | Benign              | 0.112      |
| ND5  | 12004    | G G G A          | Val - Met               | Benign              | 0.851      |
| ND5  | 12190    | A A G A          | lle - Val               | Benign              | 0.128      |
| ND5  | 12383    | G G G A          | Ala - Thr               | Benign              | 0.443      |
| ND5  | 13478    | C A C A          | Met - Ile               | Benign              | 0.083      |
| ND6  | 13581    | T G G G          | Val - Gly               | Benign              | 0.334      |
| CytB | 14161    | T T C T          | lle - Thr               | Benign              | 0.694      |
cluded that these substitutions could not change secondary structure of RNAs.

Detection of positive selection
The dN/dS(ω) values were calculated according to Yang (1997). The ratios (dN/dS) of the 4 goat lineages and 9 mammals (sheep, cow, yak, pig, horse, donkey, dog, cat, rat; Accession nos. AF010406, J01394, AY684273, AIJ002189, NC_001640, X97337, NC_002008, U20753, DQ673907) in all 13 protein coding genes were calculated (Appendix Table 2). The result indicated the base substitutions among 4 goat lineages and 9 mammals were not affected by Darwinian positive selection (ω=0.0399-0.1529). The same conclusion has also been drawn using the Nei and Gojobori method (Nei and Gojobori, 1986). Phylogenetic reconstruction of 10 mammals was performed using the mtDNA coding region sequences collected in this study and the published sequences of another 9 mammals (Figure 1).

Discussion

Estimates of divergence time
Molecular clock analyses are highly sensitive to evolutionary distance between main clades. Furthermore, the choice of an outgroup is important (Rogaev et al., 2006). The Stationary Markov Model (Saccone et al., 1990) was used to measure the sequence divergence, K [substitutions (subs)/site], of the different functional regions for each pair of species. The absolute nucleotide substitution rate, V (subs/site year⁻¹), was calculated as V=K/2T, where T is the divergence time (Pesole et al., 1999). The various functional regions of mtDNA evolved at different rates. The non-synonymous sites, D-loop central domain, and tRNA and rRNA genes changed much more slowly than the synonymous sites. The nucleotide substitutions among 7 species (goat, sheep, cow, yak, pig, horse and donkey) in 22 tRNA coding sequences and the 12S and 16S rRNA genes were calculated. The tRNA component had a slightly lower average

Table 3. Single nucleotide polymorphisms in goat mitochondrial RNA.

| Gene   | Position lineage | Variation substitution | Base | Domain                                      |
|--------|------------------|------------------------|------|--------------------------------------------|
| tRNA   | Ile 3738         | D                      | A-G  | Connection of anticodon arm and T arm      |
|        | Ala 5083         |                        |      |                                             |
| L strand | D A-G           |                        |      |                                             |
|        | Trp 4962         | B                      | A-G  | D loop                                     |
|        | Gly 9415         | D                      | A-G  | Connection of anticodon arm and D arm      |
|        | His 11567        | C                      | A-G  | Connection of anticodon arm and D arm      |
|        | Leu 11689        | B                      | G-A  | D loop                                     |
|        | Leu 11700        | B                      | A-G  | Connection of anticodon arm and D arm      |
| 12SrRNA| 165 C            | T-C                    | Loops|
|        | 205 B            | C-T                    | Loops|
|        | 211 C            | C-T                    | Loops|
|        | 292 D            | G-A                    | Loops|
|        | 1190 C           | T-C                    | Loops|
|        | 1367 C           | C-T                    | Loops|
|        | 1848 BC          | T-C                    | Loops|
|        | 2090 BC          | T-C                    | Loops|
|        | 2117 D           | T-C                    | Ustructured single strand                  |
|        | 2206 D           | T-C                    | Loops|
|        | 2415 B           | G-A                    | Loops|
|        | 2515 B           | C-T                    | Loops|
|        | 2637 D           | A-G                    | Stems (principle of G-U pairing)           |
sequence divergence than either the 12S or the 16S rRNA genes (Table 4), which was also observed in other mammalian lineages (Gu et al., 2006; Pesole et al., 1999).

The time since divergence between the goat and other mammals was based on previously observed substitution rates specific for each mammalian mtDNA functional component (Pesole et al., 1999). The time since divergence was found to be 9.53-9.86 million years between goat and sheep, 12.83-17.63 million years between goat/sheep and cow/yak, 20.55-27.69 million years between goat/sheep and pig, and 21.52-33.57 million years between goat/sheep and horse/donkey (Table 4). Gu et al. (2006) estimated that divergence between goat/sheep and cow/yak occurred at 13.14-27.99 million years before the present day. This is consistent with the data of 12.83-17.63 million years in the study.

**Complete mitochondrial DNA and molecular evolution**

The study of molecular evolution is made more difficult by the fact that only a few full nuclear genome sequences have been completed in eukaryotes and prokaryotes. Mitochondrial genomes offer a broad range of characters to study phylogenetic relationships of animal taxa. Besides nucleotide and amino acid sequences, tRNA secondary structures, rRNA secondary structures (Macey et al., 2000), deviations from the universal genetic code (Castresana et al., 1998; Telford et al., 2000), as well as changes in the mitochondrial gene order (Boore et al., 1995; Boore et al., 1998) have been successfully used as characters in phylogenetic inference. In particular, the changes in gene order prove to be extremely reliable phylogenetic characteristics because the probability that homoplastic translocations occur in closely related taxa is very low (Pesole et al., 1999).

There is increasing archaeological evidence suggesting that goats, in the form of their wild progenitor the bezoar (Capra aegagrus), were the first wild herbivores to be domesticated. The studies in literature suggest that this happened 10,000 years ago at the dawn of the Neolithic period, in the Middle East region known as the Fertile Crescent (Legge, 1996; Zeder and Hesse, 2000). Parma et al. (2003) reported that the whole mitochondrial genome sequence of goat was completed by using the available goat sequences aligned with the cattle mtDNA. The resulting gaps were then amplified by PCR using goat specific primers; the complete sequence did not belong to any lineage. In the study, the complete nucleotide sequence of four goat mtDNA lineages was sequenced. The 4 sequences represented four lineages. The goat mtDNA sequences submitted to GenBank had different lengths (16,640; 16,641 and 16,642) as a result of base insert/delete. The complete sequence of four goat mtDNA lineages will be useful for further study of goat evolution and genetics.

**Conclusions**

The goat mtDNA was 16640-16642bp in length, with the third codon position showing obvious AT bias. Eighteen non-synonymous sites were found by aligning amino acid sequence of four goat mtDNA lineages, and the non-synonymous sites did not affect the function of protein. In the region for coding tRNA and rRNA, SNP occurred in loops, unstructured single strand, and stems, which conformed with the principle of G-U pairing. It was, therefore, concluded that these substitutions could not change the secondary structure of RNAs. The ratios (dN/dS) of the four goat lineages and 9 mammals in all 13 protein coding genes were calculated and the results indicated that the base substitutions among four goat lineages and 9 mammals were not affected by Darwinian positive selection ($\omega=0.0399-0.1529$).

**References**

Anderson, S., Bankier, A.T., Barrell, B.G., De Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R., Young, I.G., 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457-464.

Anderson, S., De Bruijn, M.H., Coulson, A.R., Eperon, I.C., Sanger, F., Young, I.G., 1982. Complete sequence of bovine mitochondrial DNA: conserved features of the mammalian mitochondrial genome. J. Mol. Biol. 156:683-717.

Boore, J.L., Collins, T.M., Stanton, D., Daehler, L.L., Brown, W.M., 1995. Deducing the pattern of arthropod phylogeny from mitochondrial DNA rearrangements. Nature 376:163-165.

Boore, J.L., Lavrov, D., Brown, W.M., 1998. Gene translocation links insects and crustaceans. Nature 392:667-668.

Brown, W.M., George, M.J., Wilson, A.C., 1979. Rapid evolution of animal mitochondrial DNA. P. Natl. Acad. Sci. USA 76:1967-1971.

Castresana, J., Feldmaier-Fuchs, G., Paabo, S., 1998. Codon reassignment and amino acid composition in hemichordate mitochondria. P. Natl. Acad. Sci. USA 95:3703-3707.

Chen, S.Y., Su, Y.H., Wu, S.F., Sha, T., Zhang, Y.P., 2005. Mitochondrial diversity and phylogenographic structure of Chinese domestic goats. Mol. Phylogenet. Evol. 37:804-814.

Clayton, D.A., 1992. Transcription and replication of animal mitochondrial DNAs. Int. Rev. Cytol. 141:217-232.

Giles, R.E., Blanc, H., Cann, H.M., Wallace, D.C., 1988. Maternal inheritance of human mitochondrial DNA. P. Natl. Acad. Sci. USA 77:6175-6179.

Gu, Z.L., Zhao, X.B., Li, N., Wu, C.X., 2006. Complete sequence of the yak (Bos grunniens) mitochondrial genome and its evo-
lutionary relationship with other ruminants. Mol. Phylogenet. Evol. 42:248-255.

Gyllensten, U., Warton, D., Wilson, A.C., 1985. Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice. J. Hered. 76:321-324.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acid. S. 41:95-98.

Hassanin, A., Bonillo, C., Nguyen, B.X., Cruauad, C., 2010. Comparisons between mitochondrial genomes of domestic goat (Capra hircus) reveal the presence of umnts and multiple sequencing errors. Mitochondr. DNA 21:68-76.

Hiendleder, S., Lewalski, H., Wassmuth, R., Legge, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acid. S. 41:95-98.

Hassanin, A., Bonillo, C., Nguyen, B.X., Cruauad, C., 2010. Comparisons between mitochondrial genomes of domestic goat (Capra hircus) reveal the presence of umnts and multiple sequencing errors. Mitochondr. DNA 21:68-76.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5:150-163.

Legge, T., 1996. The origins and spread of agriculture in Eurasia. UCL Press, London, UK.

Lin, C.S., Sun, Y.L., Liu, C.Y, Yang, P.C., Chang, L.C., Cheng, I.C., Mao, S.J., Huang, M.C., 1999. Complete nucleotide sequence of pig (Sus scrofa) mitochondrial genome and dating evolutionary divergence within Artiodactyla. Gene 236:107-114.

Luikart, G., Gielly, L., Excoffier, L., Vigne, J.D., Bouvet, J., Taberlet, P., 2001. Multiple maternal origins and weak phylogeographic structure in domestic goats. P. Natl. Acad. Sci. USA 98:5927-5932.

Macey, J.R., Schulte, J.A., Larson, A., 2000. Evolution and phylogenetic information content of mitochondrial genomic structural features illustrated with acrodont lizards. Syst. Biol. 49:257-277.

Mannen, H., Nagata, Y., Tsuji, S., 2001. Mitochondrial DNA reveal that domestic goat (Capra hircus) are genetically affected by two subspecies of bezoar (Capra aegagrus). Biochem. Genet. 39:45-54.

Naderi, S., Rezaei, H.R., Taberlet, P., Zundel, S., Rafat, S.A., Naghash, H.R., El-Barody, M.A., Ertugrul, O., Pompanon, F., 2007. Large-scale mitochondrial DNA analysis of the domestic goats reveals six haplogroups with high diversity. PLoS ONE 2:e1012.

Nei, M., Gojobori, T., 1989. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3:418-426.

Nishihori, M., Hanazono, M., Yamamoto, Y., Tsuzuki, M., Yasue, H., 2003. Complete nucleotide sequence of mitochondrial DNA in chickens, White Leghorn and White Plymouth Rock. Anim. Sci. J. 74:437-439.

Olivo, P.D., de Van Walle, M.L., Laipsi, P.J., Hauswirth, W.W., 1983. Nucleotide sequence evidence for rapid genotypic shifts in the bovine mitochondrial DNA D-loop. Nature 306:400-402.

Parma, P., Felignini, M., Greggi, G., Enne, G., 2003. The complete nucleotide sequence of goat (Capra hircus) mitochondrial genome. DNA mitochondrial genome. DNA Sequence 14:199-203.

Peso, G., Gissi, C., De Chirico, A., Saccone, C., 1999. Nucleotide substitution rate of mammalian mitochondrial genomes. J. Mol. Evol. 48:427-434.

Rogaev, E.I., Moliaka,Y.K., Malashchuk, B.A., Kondrashov, F.A., Derenko, M.V., Chumakov, I., Grigorenko, A.P., 2006. Complete mitochondrial genome and phylogeny of Pleistocene mammoth Mammuthus primigenius. PLoS Biol. 4:e73.

Saccone, C., Calavei, C., Pesole, G., Preparata, G., 1990. Influence of base composition on quantitative estimates of gene evolution. Method. Enzymol. 183:570-583.

Sambrook, J., Russell, D.W., 2000. Molecular cloning: a laboratory manual. Cold Spring Harbor Lab Press, New York, NY, USA.

Telford, M.J., Herniou, E.A., Russell, R.B., Littlewoood, D.T., 2000. Changes in mitochondrial genetic codes as phylogenetic characters: two examples from the flatworms. P. Natl. Acad. Sci. USA 97:11359-11364.

Wu, Y.P., 2008. Diversity and phylogenetic relationships of some Chinese goat breeds. Chinese Academy of Agricultural Sciences Publ., Beijing, China.

Wu, Y.P., Guan, W.J., Zhao, Q.J., He, X.H., Pu, Y.B., Luo, J.H., Xie, J.F., Han, J.L., Rao, S.Q. Ma, Y.H., 2009. A fine map for maternal lineage analysis by mitochondrial hypervariable region in 12 Chinese goat breeds. Anim. Sci. J. 8:372-380.

Xu, X., Arnason, U., 1994. The complete mitochondrial DNA sequence of the horse, Equus caballus: extensive heteroplasy of the control region. Gene 148:117-123.

Yang, Z., 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. Comput. Appl. Biosci. 13:555-556.

Zeder, M.A., Hesse, B., 2000. The initial domestication of goats (capra hircus) in the Zagros mountains 10,000 years ago. Science 287:2254-2257.
# APPENDIX

Appendix Table 1. Primer sequence, corresponding polymerase chain reaction product size and position.

| Code | Primer sequence                                      | Product size | Position   | Temperature |
|------|------------------------------------------------------|--------------|------------|-------------|
| G1   | 5’ CGCCCTCCAAATCAATAA 3’<br>5’ CCCATTCTCCATCTCC 3’ | 639          | 144-782    | 53          |
| G2   | 5’ CCCATTCTCCATCTCC 3’<br>5’ TTGGCCACATGATGATGTTG 3’ | 621          | 765-1391   | 52          |
| G3   | 5’ CAACTCACTATGTGGA 3’<br>5’ TAGGGTAACTCGTGCCGT 3’ | 991          | 1373-2363  | 53.2        |
| G4   | 5’ AGTTTTGGGTTGGGGTGAC 3’<br>5’ ATAGGGCCCGTGTTAGGGG 3’ | 1405         | 2237-3641  | 56          |
| G5   | 5’ TACCCCCGATCCGGTAC 3’<br>5’ TTAGGGCTTTGAAGGCTC 3’ | 1421         | 3569-4989  | 56          |
| G6   | 5’ AACTACACCAAATCCTCAAT 3’<br>5’ CCAGTTGGGAGCGGATAAT 3’ | 1359         | 4919-6277  | 56          |
| G7   | 5’ GCACCCGGCTTACTCGAC 3’<br>5’ ATGGGTAGTACACTCCTGC 3’ | 1227         | 6225-7451  | 56.8        |
| G8   | 5’ AACCTGGAACACTGCA 3’<br>5’ GGAGTATGACCTGACCTTG 3’ | 1427         | 7399-8825  | 55          |
| G9   | 5’ ATACCAATGATGAGATGATG 3’<br>5’ AGAGATACTGGCAATGCTGTTA 3’ | 1155         | 8769-9923  | 56          |
| G10  | 5’ AACAGGATGGAAGACAATG 3’<br>5’ GGGGTGGTGTACGATGTTA 3’ | 1182         | 9903-11084 | 55          |
| G11  | 5’ CTTGAAATCCTCCTCGTGCTCT 3’<br>5’ TGAGGTGTGATAAGGATG 3’ | 1247         | 11009-12255 | 55       |
| G12  | 5’ GGTGGTATGGACGAGCAGA 3’<br>5’ GTAGGGCAATGACGCTGTG 3’ | 1148         | 12215-13362 | 58        |
| G13  | 5’ TTGGAAGGCTTTTCACAG 3’<br>5’ CTCGGTTGCGGTGATGTTG 3’ | 1289         | 13121-14409 | 54       |
| CB   | 5’ ATGATGAAAGAAGACTAC 3’<br>5’ CCAACTGATACGCTGCT 3’ | 1344         | 14106-15449 | 50       |
| DL   | 5’ AGACTCAAGGAAAGAGCCA 3’<br>5’ GATTTGAGAACGCCCTACT 3’ | 1438         | 15359-16640 | 55       |