Journal of Animal & Plant Sciences, 32(6): 2022, Page: 1724-1730
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2022.6.0580

MOLECULAR MARKER (PCR-RFLP) ASSISTED IDENTIFICATION OF MEAT SPECIES BY MITOCHONDRIAL CYTOCHROME C OXIDASE SUBUNIT I (*CO*I) GENE

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ABSTRACT

To guard consumers from meat adulteration, there is a dire obligation to encompass a sensitive, simple, and precise way for the identification of meat animal species. Molecular techniques have hoisted the expectations of developing authentic and reliable methods for testing the origin of meat species. This study explains the utilization of Polymerase Chain Reaction and Restriction Fragment Length Polymorphism along with mitochondrial cytochrome c oxidase subunit I gene to identify six meat species i.e., buffalo (*Bubalus bubalis*), cow (*Bos indicus*), cat (*Felis catus*), goat (*Capra hircus*), donkey (*Equus asinus*) and sheep (*Ovis aries*). Universal primers were utilized to amplify a specific region (approx. 710 bp) encoding the mitochondrial *CO*I gene in each species and after *insilico* analysis two restriction enzymes (*Tas*I and *Tru*I) were selected to digest all desired amplicons. Restriction analysis on 3% agarose gel uncovered unique species-specific restriction profiles. The level of *CO*I variation by utilizing *Tas*I and *Tru*I was found to be adequate to produce effectively analyzable species-specific profiles that could recognize all species unambiguously. This study suggested that PCR-RFLP is a swift and reliable scheme in favor of recognition and discrimination of analyzed meat species by mitochondrial *CO*I gene and can be employed in food control laboratories.

Keywords: PCR-RFLP; Mitochondrial *CO*I gene; Molecular markers.

Published first online June 11, 2022. Published final November 20, 2022

INTRODUCTION

Livestock being a critical sub-sector of agribusiness performs a significant job in the lives of farmers in developing nations like Pakistan. This area not just guarantees food security, but also expands the per capita income of poor, landless, and subsistence dimension of farmers, yet additionally filling the GDP of the nation (Anonymous, 2017). Meat is one of the major byproducts of livestock and an important source of high-quality protein and amino acids for daily requirements. Pakistani meat has a novel taste because of its natural nature and has been sent out fundamentally to gulf marketplaces in extensive amounts (Ayyub *et al*., 2011). The meat demand has radically expanded during the last decade because of quick urbanization, economic development, industrialization, and information about balanced eating regimens (Anonymous, 2017). The raising meat export has likewise marked down its accessibility for local consumers that have added to meat cost increase and this tendency is continually in progress.

Admixing of nutritious meat with noxious one is a great concern in retail markets (Wong *et al*., 2010, Njaramba *et al*., 2021). Additionally, meat adulteration has raised an alarming situation as the meat of some animals is considered Haram in the religion of Islam like porcine animals and some others. Previously numerous investigative approaches like immune assays, electrophoresis, chromatography, etc. were extensively employed to classify defilement in meat items (Waugh, 2007, Abbas *et al*., 2018), however; due to certain constraints of these methods (i.e., rapid degradation of soluble proteins, restricted availability of cross-reactive free antibodies and presence of proteins are related to age, maturity, and type of tissue) molecular techniques are being preferred (DeMasi, 2015).

DNA-based techniques have provided authentic and consistent ways for the origin of meat species identification mainly due to the stability of DNA with conditions like pressure, temperature, and chemical treatments (Arslan *et al*., 2006). Moreover, it is there in majority of cell types and its structure is conserved in every tissue. Predominantly, mitochondrial DNA (mtDNA) has been one of the extensively premeditated regions in eukaryotes that performed a salient job in the progress of specie identification (Erwanto *et al*., 2012).

PCR based systems have successfully employed different mtDNA genes, for example, cytochrome oxidase subunit I (*CO*I) (Haider *et al*., 2012), the mitochondrial D-loop region (Babar *et al*., 2015), cytochrome b (Castello *et al.*, 2004), 16S rRNA (Dalmasso *et al.*, 2004), and 12S rRNA (Girish *et al*., 2005) for diagnostic studies of animals and make the amplification a fairly sensitive procedure.

Mitochondrial DNA restriction fragment length polymorphism (mtDNA-RFLP), species-specific repeats (SSR), random amplified polymorphic DNA PCR (RAPD-PCR), real-time PCR, multiplex PCR, and DNA sequencing are the present-day procedures that permit the identification of meat species (Cheng *et al*., 2014; Farag *et al*., 2015). Haunshi *et al*., (2009) suggested that compared to other methods for species identification, RFLP is a quick, economical and simple technique. This strategy is grounded on the intensification of a DNA fragment of the anticipated gene by PCR and pursued by its processing with suitably chosen restriction enzymes that provide precise species profiles (Pascoal *et al*., 2005).

The investigators who are aimed to deal with the identification of meat species pay special attention to the *CO*I gene as the DNA sequence of this gene has the adequate variability to differentiate between species of fish, birds, some butterflies, and spiders (Dybus and Knapik, 2005). *CO*I is a catalytic subunit of the enzyme of mitochondrial DNA and delegated as a region of barcoding for the Kingdom Animalia (Haider *et al*., 2012). Thus, the aim of this study was to inquire about the use of PCR-RFLP along with the mitochondrial *CO*I gene to identify the tissue origin of six animal species i.e., buffalo (*Bubalus bubalis*), cow (*Bos indicus*), cat (*Felis catus*), goat (*Capra hircus*), donkey (*Equus asinus*) and sheep (*Ovis aries*).

MATERIAL AND METHODS

Sample collection: A total of 50 blood and meat samples of buffalo (10), cow (10), goat (10), and sheep (10) were collected from different butcher shops in Lahore whereas cat (5) and donkey (5) meat samples were collected from Pet Centre and Outdoor Clinic of University of Veterinary and Animal Sciences (UVAS) Lahore, Pakistan. A single muscle tissue sample from a dead cat and two muscle tissue samples from dead donkeys were collected from UVAS. The remaining samples of these species were of blood which was also collected from UVAS Pet Centre and Outdoor Clinic. All the collected samples were taken to the Molecular Biology Laboratory of the Institute of Biochemistry and Biotechnology, UVAS Lahore, and stored at -20 ^{o}C till further processing. Dog (*Canis lupus familiaris*) was not included in the study due to failure in amplification of its DNA sample. For blood samples, 50-200 µl of blood was taken carefully into EDTA (0.5 M with pH 8.0) containing vials whereas meat samples were taken and placed in sterile test tubes and stored at a temperature of -20 degrees centigrade until isolation of DNA.

Extraction of DNA: Extraction of DNA from blood (i.e., 50 µl) and meat samples (i.e., 200 mg) was performed following procedures described previously (Matsunaga *et al.*, 1999). Moreover, for quality and concentration determination of DNA all the extracted DNA specimens were quantified through a Thermo Scientific NanoDrop ND-1000 at 260/280 nm.

Polymerase chain reaction (PCR): The PCR amplification for the mitochondrial *CO*I gene was employed for all DNA samples of the six animal species. The reported universal *CO*I primers employed here were: Forward: 5'-GGTCAACAAATCATAAAGATATTGG-3' and Reverse: 5'-TAAACTTCAGGGTGACCAAAAAATCA -3' (Folmer *et al.,* 1994). For PCR, a reaction volume of 25 µl was made in a PCR tube containing 2.5 µl of 10× PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 2 µl of 2.5 mM deoxynucleotide triphosphate (dNTPs), 0.25 µl of 5U *Taq* DNA polymerase, 0.5 µl of 10 pico-moles of each primer (forward primer and reverse primer) and 2 µl of the target DNA. The 25 µl reaction volume was obtained by the addition of ddH_2O. The PCR machine was programmed for initial denaturation at 95°C for 5 min followed by 5 cycles of holding at 94°C for 30 s, at 45°C for 30 s, and at 72°C for 45s. Another 30 cycles were programmed for annealing at 50°C for 30 s instead of 45°C. There was a step of final extension at 72°C for 10 min followed by a hold at 4°C for infinity. The reliability of the PCR products obtained by amplification of the targeted gene was confirmed through positive as well as negative controls. All the amplified products were analyzed on a 1.5% agarose gel while resolved PCR products were visualized by placing the gel on a gel documentation system (Eagle eye gel documentation system, Stratagene, USA).

PCR-RFLP: For PCR-RFLP analysis, an *in-silico* investigation recognized a few endonucleases that ought to demonstrate help for recognizing among the animal species screened. Of these, *Tas*I and *Tru*I (Thermo Scientific) were chosen for the digestion of *CO*I gene amplicons. A sum amount of 30 µl of the reaction mixture was made in a PCR tube containing 10 µl of PCR product, 2 µl of PCR buffer, 0.5 µl of the enzyme, and 17.5 µl of ddH_2O. The restriction was executed for 2 hours at 65°C followed by running of digested PCR products on a 3% agarose gel along with a 100 bp standard GeneRuler DNA Ladder (Thermo Scientific) and pictured in a gel documentation system under UV light.

RESULTS AND DISCUSSION

Blood and meat samples were collected from the selected species from which genomic DNA was extracted and electrophoretically analyzed on a 0.8% agarose gel. The presence of DNA in all samples was disclosed under UV light which showed DNA bands of high molecular weight. The concentration of DNA was calculated spectrophotometrically and ranged from 20-200 ng/µl. An optical density was obtained at 260 and 280 nm which revealed that extracted DNA was of very good quality as the ratio of readings ranged from 1.7 to 1.9. PCR amplification of *CO*I gene segment was done with the help of universal forward and reverse primers that perfectly amplified a fragment of approx. 710 bp of the mitochondrial *CO*I gene is shown in Figure 1.

Amplified PCR fragments obtained from mitochondrial *CO*I gene from each buffalo, cow, cat, goat, donkey, and sheep were digested with appropriate restriction enzymes *Tas*I and *Tru*I. Digestion of each PCR product with restriction enzymes generated specific DNA patterns in all meat species (Table 1 & Figure 2; Also see supplementary Figure 1).

In cow, a band pattern defined by a major fragment of 213 bp with *Tas*I and the other band pattern defined by a major fragment of 531 bp with *Tru*I was observed. In donkey, a band pattern defined by major fragments of 244 bp and 400 bp were observed by *Tas*I and *Tru*I restriction enzymes respectively. In the cat, a band pattern defined by a major fragment of 244 bp with *Tas*I and the other band pattern defined by a major fragment of 511 bp with *Tru*I was observed. In buffalo, a band pattern defined by a major fragment of 230 bp with *Tas*I and the other band pattern defined by a major fragment of 463 bp by *Tru*I was observed. In goat, a band pattern defined by a major fragment of 213 bp with *Tas*I and the other band pattern defined by a major fragment of 463 bp with *Tru*I was observed. In sheep, a band pattern defined by a major fragment of 388 bp with *Tas*I and the other band pattern defined by a major fragment of 388 bp by *Tru*I was observed. Although having unique restriction profiles, the major banding pattern of goat, sheep, and cow was the same for *Tas*I i.e., 213 bp thus cannot be utilized to differentiate among them but it can be used to differentiate these species in comparison to buffalo and donkey. *Tas*I can also be used to differentiate among cow, buffalo, and cat meats by producing different sizes of major bands. In comparison to *Tas*I, *Tru*I was found to be more probative to discriminate among all analyzed meat species as the major banding pattern of the donkey, cow, goat, sheep, and the cat was different from *Tas*I i.e., 400 bp, 531 bp, 463 bp, 388 bp, and 511 bp, respectively. Although goat and buffalo samples produced the same size of major bands after *Tas*I endonuclease treatment i.e., 463 bp, their complete digestion banding pattern was unique for each meat type and thus can also be used to differentiate between buffalo and goat meat.

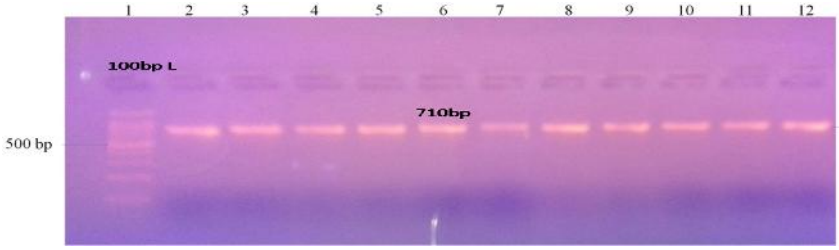

Figure 1. PCR amplification of *CO*I gene for six animal species samples. Lane 1: A DNA ladder of 100 bp; Lanes 2-3, 4-5, 6-7, 8-9, 10-11, and 12 represent PCR amplified products of the cow, buffalo, goat, sheep, donkey, and cat, respectively.

Table 1: Banding pattern of different species (in bp) with *TasI* and *TruI* endonucleases.

Enzymes	Cow	Buffalo	Goat	Sheep	Donkey	Cat
TasI	213	231	213	213	245	245
	165	213	165	165	139	229
	103	95	84	84	120	198
	84	76	80	78	95	36
	66	36	63	63	93	
	36	27	60	60	16	
	30	16	27	27		
	11	14	16	16		
TruI	531	462	462	388	230	542
	103	246	166	225	210	166
	74		59	74	180	
			21	21	88	

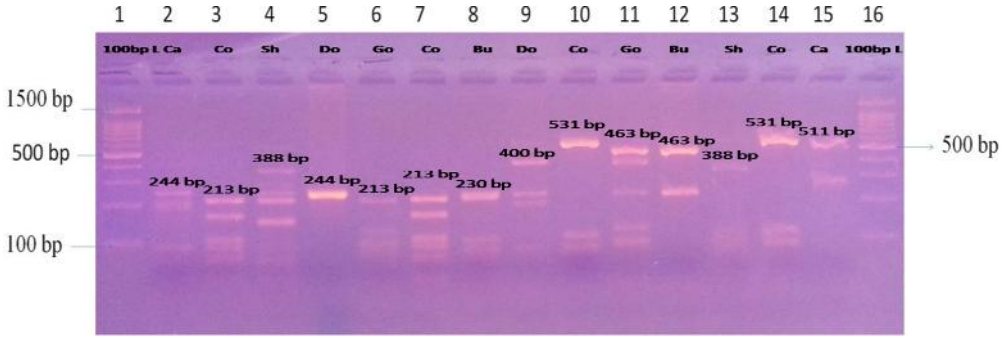

Figure 2. Restriction profiles of *CO*I amplicons using *Tas*I and *Tru*I enzymes for the six meat species. Lane 1 and 16 (100 bp DNA ladder), lanes 2-8 restriction profiles combination yielded for cat, cow, sheep, donkey, goat, cow, and buffalo samples with enzyme *Tas*I, lanes 9-15 restriction profiles combination yielded for donkey, cow, goat, buffalo, sheep, cow, and cat with enzyme *Tru*I. Some patterns in Figure 2 may vary from the restriction map given in Supplementary Figure 1 due to the presence of pseudogenes or heteroplasmy.

Meat adulteration has been an issue for a long time. Accurate identification of the specie origin of meat has huge importance from social, economic, and religious points of view. Genome-based strategies by large give preferred determination proof over the conventional morphological-based techniques. This study inquiry about the use of PCR-RFLP along with mitochondrial cytochrome c oxidase subunit I (*CO*I) gene to identify the origin of six meat species i.e., buffalo (*Bubalus bubalis*), cow (*Bos indicus*), cat (*Felis catus*), goat (*Capra hircus*), donkey (*Equus asinus*) and sheep (*Ovis aries*). Numerous scientists successfully employed PCR-RFLP assay along with mitochondrial genes to categorize meat species Dalmasso *et al.* used this technique for the detection of poultry, ruminant, swine, and fish meats by targeting tRNA-Val, 12S rRNA, and 16S rRNA genes (Dalmasso *et al*., 2004). Haider *et al*. used seven endonucleases and *CO*I gene sequences to identify raw meat of bovine, turkey, chicken, pig, sheep, donkey, and camel using PCR-RFLP (Haider *et al*., 2012). Based on the mitochondrial D-loop region Montiel *et al*. identified swine meat in mixtures of heated and dry-cured meat products and discriminated pork and wild boar with *Ava*II restriction analysis (Montiel-Sosa *et al*., 2000). Mane *et al*., 2009 detected chicken meat with the use of primers pair based on the D-loop gene of mitochondria followed by restriction digestion with *Hae*III and *Sau3* enzymes (Mane *et al*., 2009). In addition to that, the ATPase gene was successfully used by Colgan *et al*. for the detection of bovine, ovine, poultry, and porcine species in bone meal and meat (Colgan *et al*., 2001). Castello *et al.* also used PCR assay for the detection of porcine and bovine by employing mitochondrial cytochrome b, ATPase 6, and ATPase 8 genes (Castello *et al*., 2004). Arslan *et al*. used a species-specific PCR technique for the recognition of sources of different meat species i.e., goat, porcine, sheep, bovine, cat, dog, and horse in mixed meat samples (Arslan *et al*., 2006). Walker *et al*. used an SYBR green-based real-time PCR to identify as well as quantify DNA from species like equine, canine, pig, guinea, feline, rat, rabbit, and hamster (Walker *et al.*, 2004). Bellagamba *et al*. used a TaqMan real-time PCR to detect meat and meat products of species like sheep, goat, swine, chicken, and cattle (Bellagamba *et al*., 2006). To avoid detection of nonspecific amplification and allow detection in multiplex reactions, Lopez-Andreo *et al*., 2006 using real-time PCR in addition to melting curve analysis for species detection. Based on Mitochondrial 12S rRNA (Lopez-Andreo *et al*., 2006), Dooley *et al*., 2004 used a TaqMan real-time PCR for the detection of admixed meat of beef, turkey, chicken, lamb, and pork (Dooley *et al*., 2004). The method in which biological tag is used is proved to be authentic for the differentiation of southern red buck meat and bovine meat by Dalton and Kotze (2001). So, it can be determined that the flesh of buffalo (*Bubalus bubalis*), cow (*Bos indicus*), cat (*Felis catus*), goat (*Capra hircus*), donkey (*Equus asinus*), and sheep (*Ovis aries*) can be distinguished qualitatively by strong and dependable PCR-RFLP of the mitochondrial *CO*I gene. Moreover, further scanning for proper restriction enzymes to identify a wide range of meat species in a single reaction may be done in the future.

Conclusion: With the passage of time awareness about the composition of food among consumers and consumption of animal protein has increased so it suits vital to rightly verify the food source. By using methods that are DNA-based our sensitivity and specificity of detection have risen. PCR-RFLP being an advanced technique of DNA-based methods exposes adulteration and species included in debasement as well as measures the level of deterioration. The use of the proposed methodology will help food authorities to more readily screen the quality of market meats and meat items subsequently reducing the rise of the new threat of adulteration.

Conflict of interest: The author states that there is no conflict of interest.

Acknowledgment: The corresponding author is obliged to the Department of Biotechnology, the University of Sargodha for funding this manuscript's publication.

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Supplementary Material

Supplementary File

Bubalus bubalis

CTCAACCAACCATAAAGATATCGGCACCCTGTACTTGCTGTTTGGTGCCTGAGCTGGCATAGTAGGGACAG
CCCTAAGCCTGCTGATTCGCGCCGAATTAGGTCAACCTGGGACCCTACTCGGAGATGACCAAATCTACAAC
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ACTTAGCAGGTGTCTCCTCAATTTTAGGGGCTATTAATTTTATTACAACAATTATCAATATAAAACCTCCTG
CAATATCACAATACCAAACCCCTTTATTCGTGTGATCCGTAATAATCACCGCCGTGCTATTACTCCTTTCAC
TTCCTGTGCTAGCAGCTGGCATTACAATGCTACTAACAGATCGAAACCTAAACACAACTTTCTTTGATCCA
GCGGGGGGAGGAGATCCTATTCTATACCAACACTTATTTTGATTCTTCGGACACCCTGAAGTATA

Bos indicus

CTCAACCAACCATAAAGATATTGGTACCCTTTACCTACTATTTGGTGCTTGGGCCGGTATAGTAGGAACAG
CTTTAAGCCTTCTAATTCGCGCTGAATTAGGCCAACCCGGAACTCTGCTCGGAGACGACCAAATCTACAAC
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CAGGAGGAGGAGATCCTATTCTATACCAACACTTATTCTGATTCTTTGGACACCCCGAAGTCTA

Capra hircus

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CCTTGAGCTTACTAATTCGCGCCGAACTAGGTCAACCCGGAACCCTACTTGGAGATGACCAGATCTACAAT
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TCCTGTATTAGCAGCTGGCATCACAATACTACTAACAGACCGAAACCTAAACACAACCTTCTTTGACCCAG
CAGGAGGAGGAGACCCTATTTTATATCAACACCTATTCTGATTCTTTGGACACCCTGAAGTATA

Ovis aries

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ACCTGGCAGGTGTCTCTTCAATTCTAGGAGCCATTAATTTTATTACAACTATTATTAATATAAAACCCCCTG
CGATGTCACAGTATCAAACCCCCTTGTTTGTATGATCTGTACTAATTACTGCCGTACTTCTCCTTCTCTCACT
TCCTGTATTAGCAGCTGGTATCACAATACTACTAACGGACCGAAACCTGAATACAACCTTTTTTGACCCAG
CAGGAGGAGGAGACCCTATCCTATATCAACACCTATTCTGATTCTTTGGGCACCCTGAAGTATA

Felis catus

TTCAACTAATCACAAAGATATTGGTACTCTTTACCTTTTATTCGGTGCCTGAGCTGGCATGGTGGGGACTGC
TCTTAGTCTTCTAATCCGGGCCGAACTGGGCCAACCTGGTACACTACTAGGAGATGATCAGATTTACAATG
TAATCGTCACTGCCCATGCTTTTGTAATGATCTTTTTTATGGTGATGCCTATTATAATTGGAGGGTTCGGAA
ACTGATTGGTCCCACTAATAATTGGAGCTCCTGACATAGCATTTCCCCGAATAAACAACATGAGCTTCTGA
CTCCTCCCTCCATCCTTTCTACTCTTACTCGCCTCATCTATGGTAGAAGCCGGAGCAGGAACTGGGTGAACA
GTATACCCACCCCTAGCCGGCAACCTGGCTCATGCAGGAGCATCCGTAGACCTAACTATTTTTTCACTACA
CCTGGCAGGTGTCTCCTCAATCTTGGGTGCTAATAATTTCATTACTACTATTACTAATATAAAACCTCCTGC
CATGTCCCAATATCAAACACCTCTATTTGTCTGATCAGTCTTAATCACTGCTGTCTTACTACTTCTATCACTT
CCAGTCTTAGCAGCGGGAATCACTATATTACTAACAGATCGAAACCTAAACACCACATTCTTTGACCCCGC
TGGGGGAGGAGATCCTATCTTATACCAACACTTATTCTGATTCTTTGGCCATCCAGAAGTTTA

Equus asinus

TTCAACTAACCACAAAGACATCGGCACTCTGTACCTCCTATTCGGCGCTTGAGCTGGAATAGTAGGAACCG
CCCTAAGCCTCCTAATCCGTGCTGAATTAGGTCAACCTGGGACTCTTCTGGGAGATGATCAGATCTACAAT
GTTATTGTAACTGCCCATGCATTCGTAATAATCTTCTTCATAGTTATACCCATCATGATCGGAGGATTTGGG
AACTGATTAGTTCCCTTAATAATTGGAGCACCCGATATAGCCTTTCCCCGAATAAACAACATAAGCTTCTG
ATTACTTCCCCCATCATTCCTACTTCTTCTTGCTTCCTCAATAATTGAAGCAGGCGCTGGAACAGGCTGAAC
CGTATATCCTCCCCTAGCTGGAAACCTAGCGCACGCAGGGGCTTCTGTTGACTTAACCATCTTCTCCCTTCA
CCTAGCTGGTGTATCCTCAATTTTAGGTGCCATCAATTTCATTACCACAATCATCAACATAAAACCACCAG
CCCTGTCCCAGTATCAAACCCCTCTATTCGTTTGATCCGTCCTCATTACGGCAGTACTCCTTCTCCTAGCTCT
TCCAGTCCTAGCAGCAGGTATTACTATGCTTCTCACAGACCGTAACTTAAACACCACCTTCTTCGACCCTGC
AGGGGGAGGAGATCCAATCCTTTACCAACACCTATTCTGATTTTTCGGACACCCTGAAGTCTA

Figure SII. Restriction map amplicon digestion by *Tas*I and *Tru*I restriction enzymes. In yellow color are presented restriction sites of *Tas*I and in red color are presented restriction sites of *Tru*I.