Interreg - IPA CBC

EUROPEAN UNION

Greece - Republic of North Macedonia

PAPESHE

Deliverable 3.1.2 Genetic characterization of the breed and genotype analysis

Project acronym: **PAPESHE**

Project full name: **Protection of Autochthonous populations of PElagonia SHEep breed in the cross-border area**

Start date of project: **30 July 2018**

Duration of project: **24 months**

Project website: **http://papeshe.vet.auth.gr/**

ARISTOTLE UNIVERSITY OF THESSALONIKI

H.A.O. "DEMETER"

ΓΕΩΤΕΧΝΙΚΟ ΕΠΙΜΕΛΗΤΗΡΙΟ ΕΛΛΑΔΑΣ

ДОЛНЕНИ

Thessaloniki, 2020

Key information

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Introduction

During the last years there has been growing scientific interest in the use of genomic information as an additional tool in conventional sheep breeding schemes. Identifying gene polymorphisms at quantitative trait loci (QTL) that may be associated with economically important trait, is the key in this effort. Polymorphisms affecting production traits (e.g. milk yield) are of great importance.

Gene assisted selection may be even more important for the genetic improvement of reproductive or health traits, since these are traits with low heritability and sometimes difficult to record, and hence their genetic improvement with conventional breeding can be laborious and not so effective.

Sheep breeding is the major activity in the Greek animal production and is very important for the rural economy. Although litter size is one of the most important traits influencing profitability of sheep production, the litter size of almost all Greek sheep breeds, except the Chios breed, is low. The Florina (Pelagonia) breed is farmed in the mountainous less favoured areas of West Macedonia (Greece). The breed has resistance to disease, perfect adaptability to the harsh environmental conditions, but prolificacy is low with ewes usually producing single lambs. Introduction of a prolificacy gene into non-prolific sheep breeds having other desired traits may effectively increase the reproductive performance of the local Greek breeds. In addition, the introduction of a major gene for prolificacy into a flock has the advantage that the gene can be introduced while retaining the breed's characteristics. Improvement of reproductive traits such as prolificacy in Greek sheep breeds would be of big value, as small increases in litter size can equal large gains in profit. Therefore, future efforts should focus on improving productivity of Greek sheep breeds through incorporation of fecundity genes responsible for increasing ovulation rate and litter size. Such a study would have important implications for the Greek sheep industry.

Recent studies have reported that the high prolificacy in many prolific sheep breeds around the world, is the result of the *FecB* gene mutation in the bone morphogenetic protein receptor IB (*BMPRIB*), or due to the *FecXI* mutation in the bone morphogenetic protein 15 (*BMP15*) gene. Newly developed DNA tests have encouraged researchers to screen for the presence of these mutations in many local sheep breeds around the world. This study extends the investigation of the *FecB* and *FecXI* mutations to Greek Florina (Pelagonia) sheep breed.

Therefore, the aim of this study was to investigate the presence of the *FecB* and *FecXI* mutations in this breed and to investigate the possibility of introgression of these genes into the Pelagonia breed, in order to enhance the reproductive rate.

Methodological approach

In order to perform the genetical characterization and the genotype analysis of the Pelagonia sheep breed, the first step was to identify the optimal conditions for the amplification of the selected genes. In order to achieve this, blood was collected and DNA was extracted from female Pelagonia breed sheeps from farms of Florina and Giannitsa. The DNA extraction and the PCR amplification of the selected genes were performed in the Laboratory of Physiology of Reproduction of Farm Animals, School of Agriculture, AUTH. The appropriate primers were designed for the amplification of the selected genes and PCR was performed, in order to identify the optimal conditions and the identification

of each individual genotype. Since this was achieved, the next step was to apply this process in the total of animals.

A total of 260 ewes were used in the study. The ewes were maintained in various farms of West Macedonia, Greece, and they were kept under the same feeding conditions: permanent sheep barns, except pasture in the afternoon. The experimental ewes were of similar age (2-2.5 years) and body conditions (65-70 kg).

Genomic DNA was extracted from ewes of Pelagonia breed from whole blood using the NucleoSpin Tissue kit and kept at -20°C until analysed. The integrity of the DNA samples was examined by electrophoresis through a 2% agarose gel.

For the investigation of the *FecB* mutation approximately 100ng of genomic DNA were amplified by PCR using a modified forced RFLP method. The reverse primer has been engineered to introduce a point mutation such that PCR products from FecB carriers contain an *Ava*II restriction site (G|GACC), whereas products from non-carriers of the mutation lack this site. The DNA was amplified in a 20μl reaction volume using the primer pair: 5-CCAGAGGACAATAGCAAAGCAAA-3, and 5-CAAGATGTTTTCATGCCTCATCAACACGGTC-3. The amplification was carried out using 35 cycles at 94°C for 15 s, 60°C for 30 s and 70°C for 30 s, followed by 72°C for 5 min. The 190 bp products were digested with *Ava*II (New England BioLabs) overnight at 37°C and the resulting products were separated by electrophoresis on a 3.5% agarose gel and visualised with ethidium bromide. Using this method, products containing the *FecB* mutation should yield 160 bp and 30 bp fragments, whilst non-carrier products remain uncut at 190 bp.

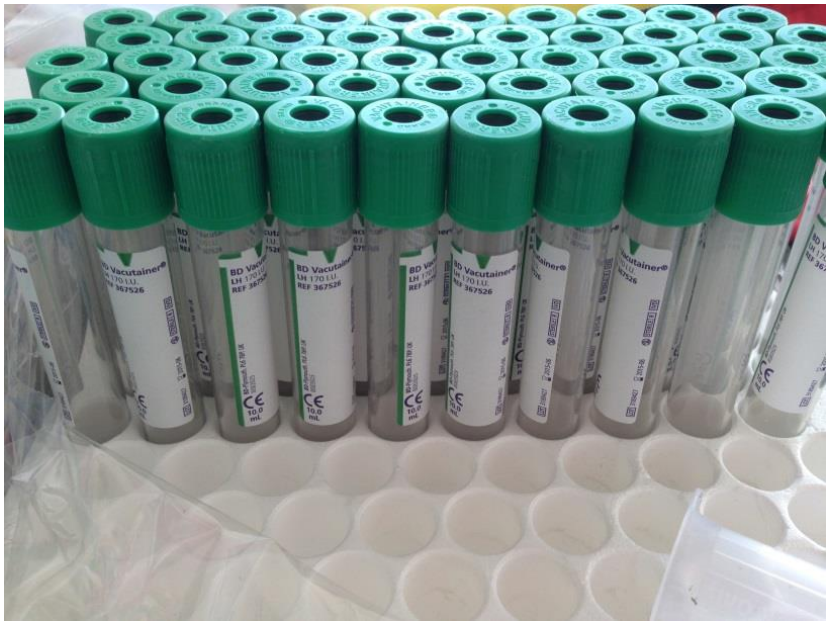
For the investigation of the *FecXI* mutation approximately 100ng of genomic DNA were amplified by PCR using the forced RFLP method. The forward primer has been designed to generate a forced *Xba*I restriction enzyme site (T|CTAGA) in PCR products from carriers of the *FecXI* mutation in the *BMP15* gene, whereas products from non-carriers of the mutation lack this site. The DNA was amplified using the primer pair 5-GAAGTAACCAGTGTTCCCTCCACCCTTTTCT-3 and 5-CATGATTGGGAGAATTGAGACC-3. The amplification was carried out using 35 cycles at 94°C for 30 s, 60°C for 40 s and 70°C for 30 s, followed by 72°C for 5 min. The 154 bp product was digested with *Xba*I (New England BioLabs) overnight at 37°C, and the products were separated by electrophoresis on a 3.5% agarose gel and visualised with ethidium bromide. Using this method, products containing the *FecXI* mutation should yield 124 bp and 30 bp fragments, whilst non-carrier products remain uncut at 154 bp.

Once genotypes were determined, allelic frequencies at each gene locus were calculated by gene counting. Deviations from Hardy–Weinberg equilibrium were examined for each locus using chi-squared tests.

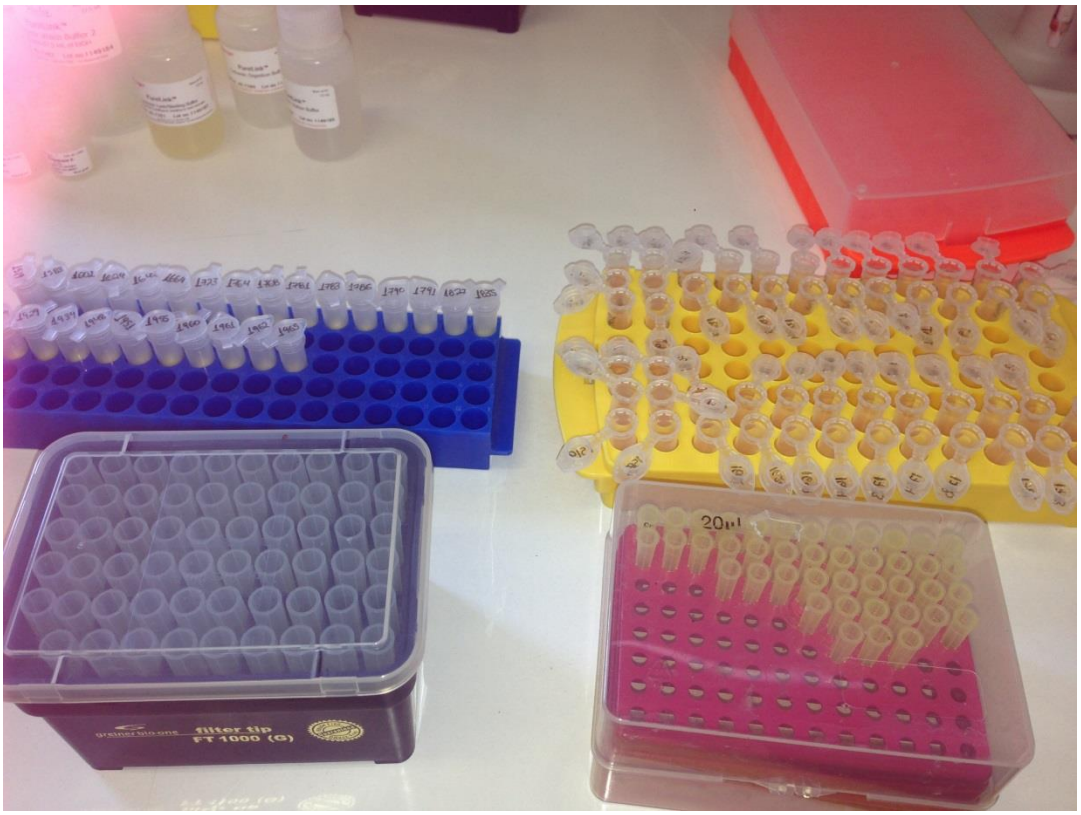
PCR Protocol	
10x Buffer	2,5μl
dNTPs	0,5μl
Forward primer	0,5μl
Reverse Primer	0,5μl
DNA	1μl
Taq Polymerase	0,25μl
dsH_2O	19,75μl
Finale volume	**25μl**

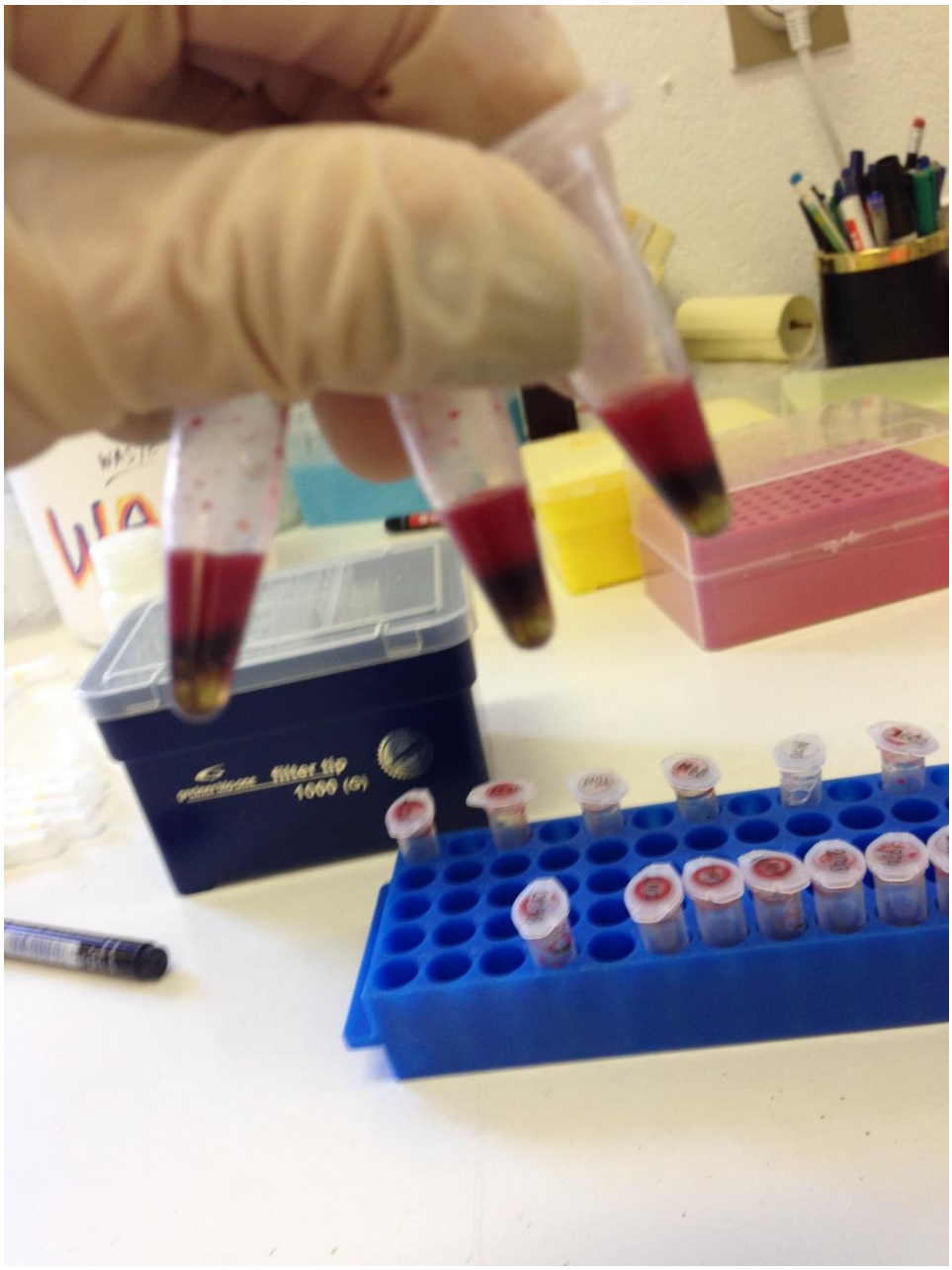
RFLP-PCR Protocol	
DNA (PCR Product)	10μl
10x Buffer	2 μl
Restriction enzyme	0.5 μl
dsH2O	7.5 μl
Finale volume	**20μl**

Blood collection from ewes.

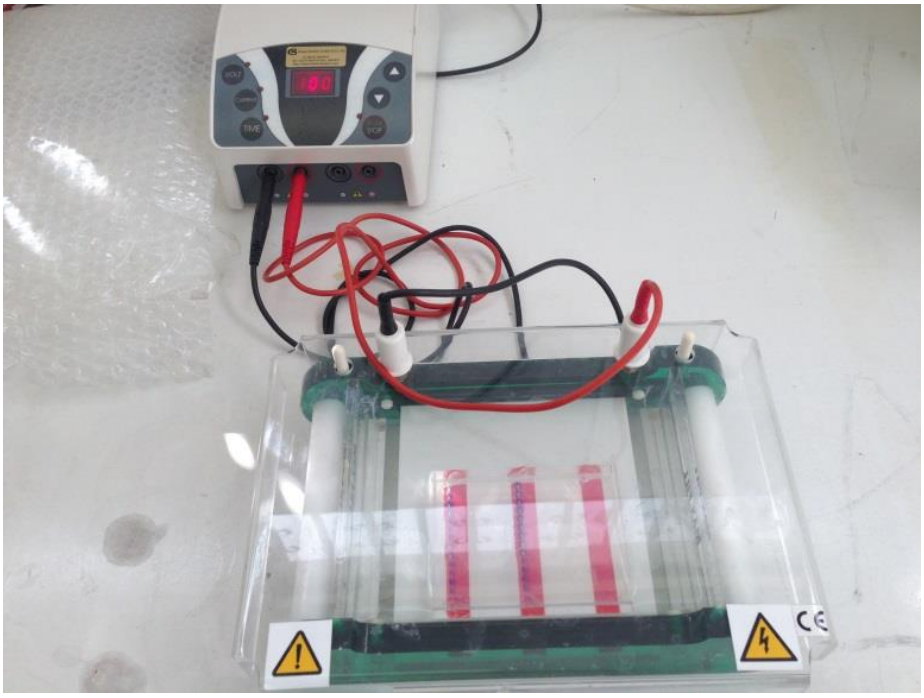


Blood collection tubes.


filter tip
FT 1000 (G)
20µl



DNA extraction reagents and tubes.

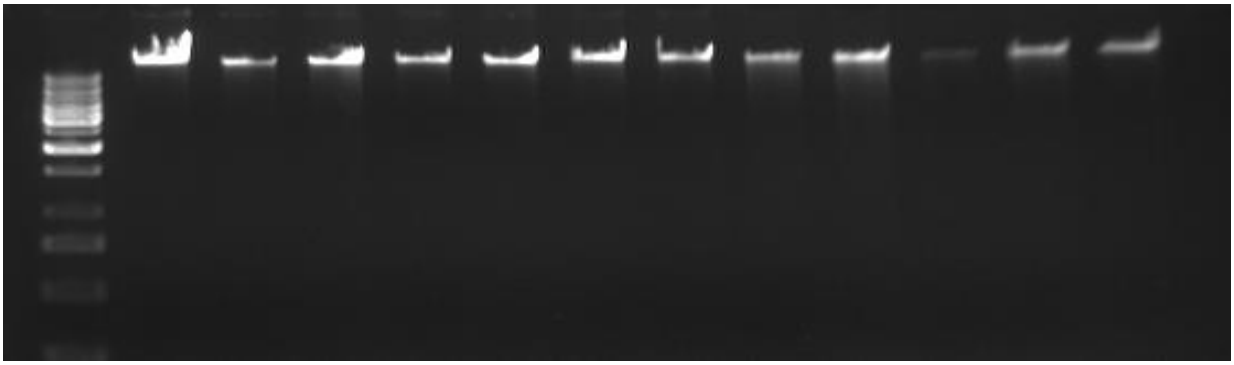


DNA electrophoresis of PCR products.

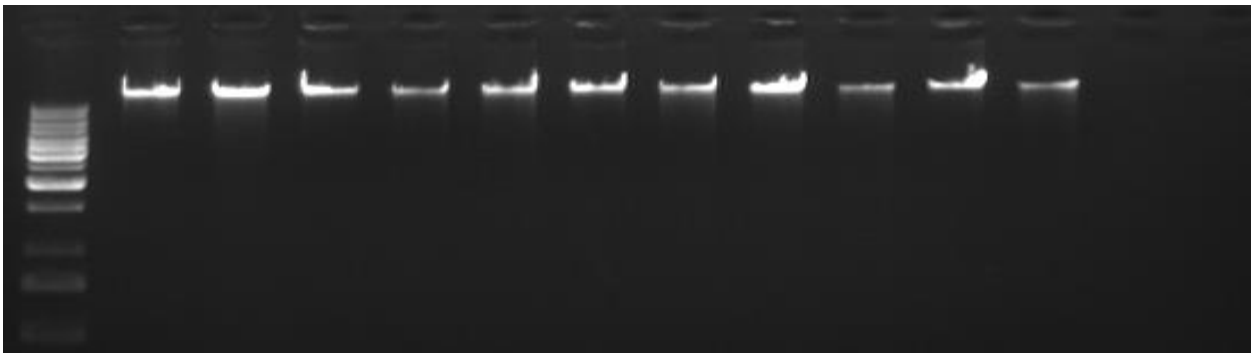
Results and Discussion

Mutations in single genes that have major effects on ovulation rate have been identified in multiple lines of sheep. In the present study mutations of the FecB and FecXI genes were investigated.

Examination of the extracted DNA from the sheep blood samples was performed by agarose gel electrophoresis, as illustrated in the indicative pictures.



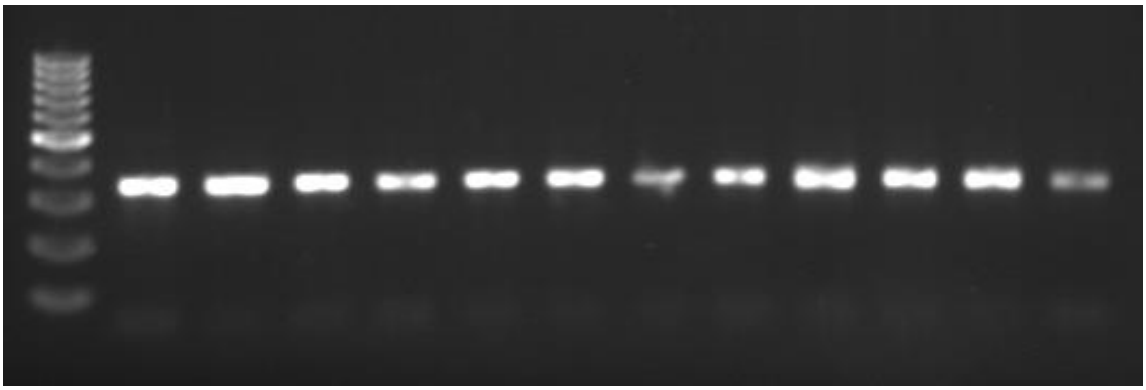
Genomic DNA from Pelagonia sheeps blood samples

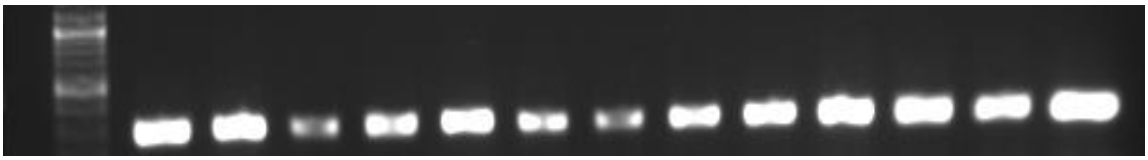


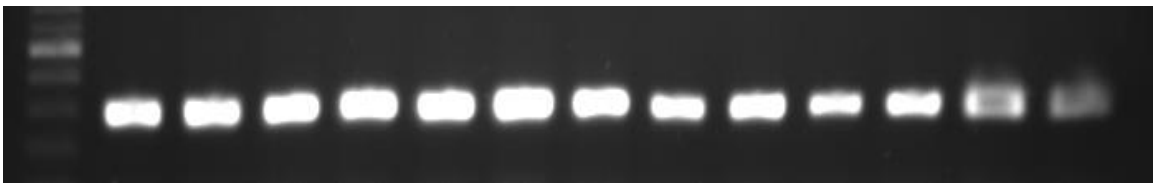
Genomic DNA from Pelagonia sheeps blood samples

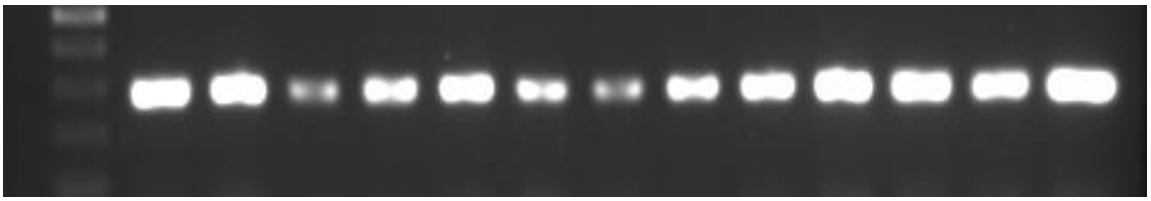
Following DNA extraction, amplification of the selected genes was performed by PCR and agarose electrophoresis, as detailed in the methodological approach section.

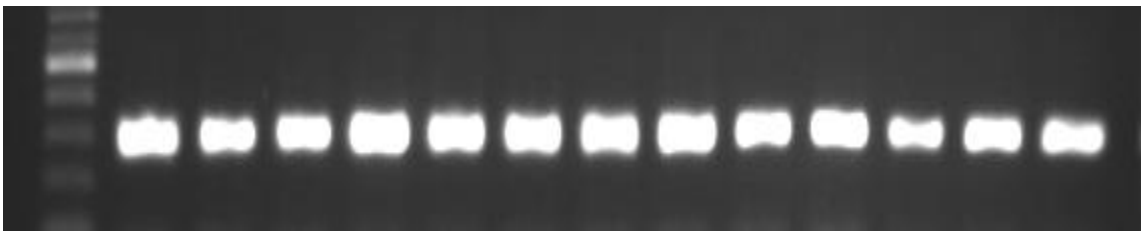
Below are indicative pictures from agarose gel electrophoresis of the PCR products, visualised with ethidium bromide under UV.

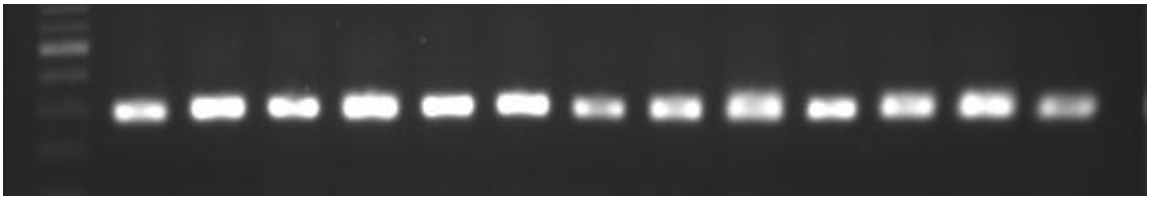


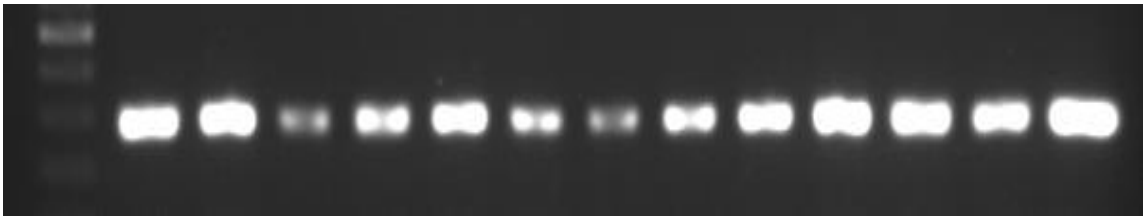


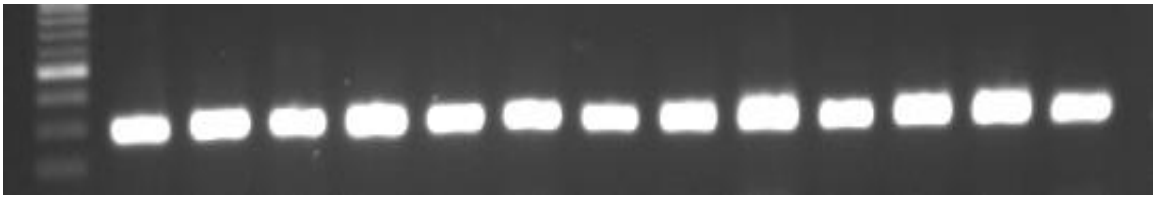


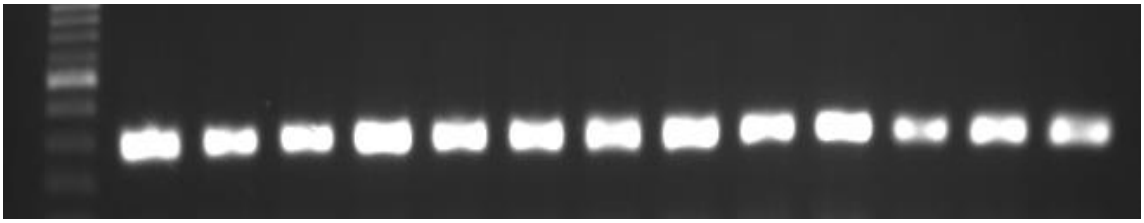


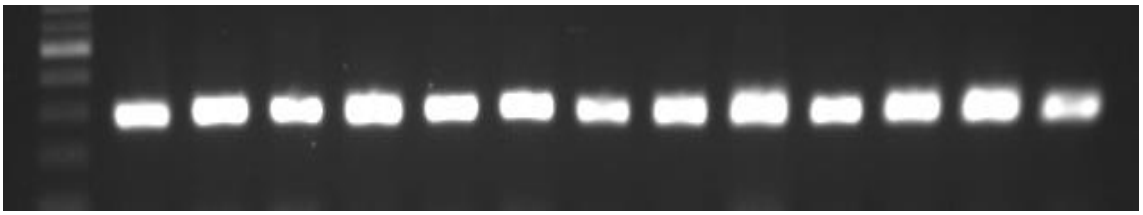


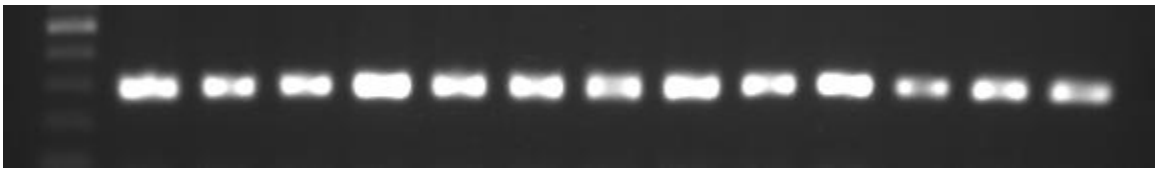


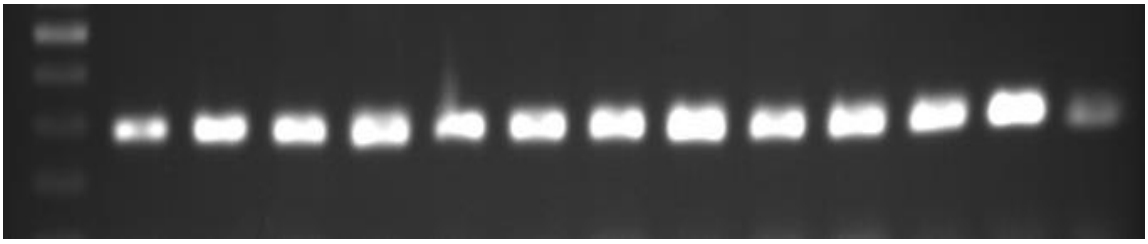


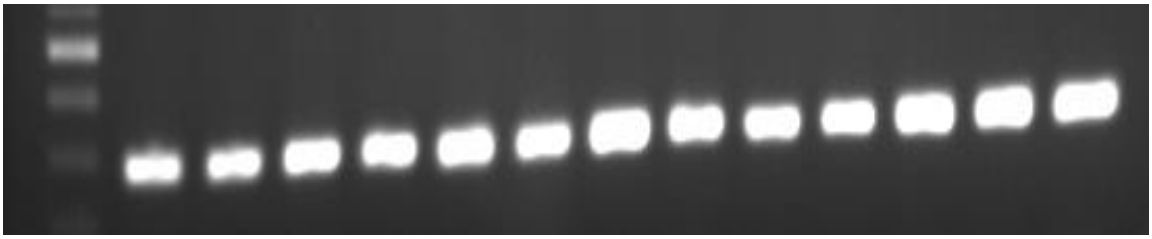


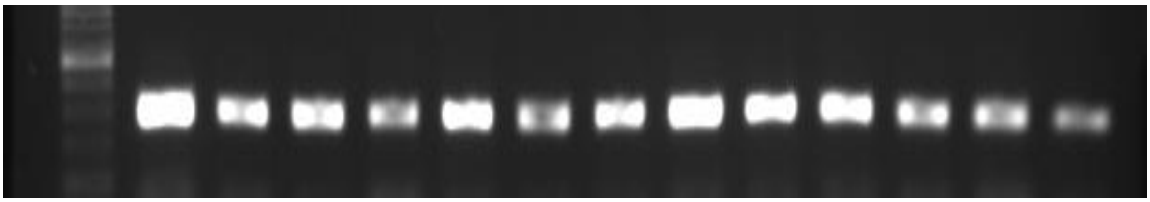


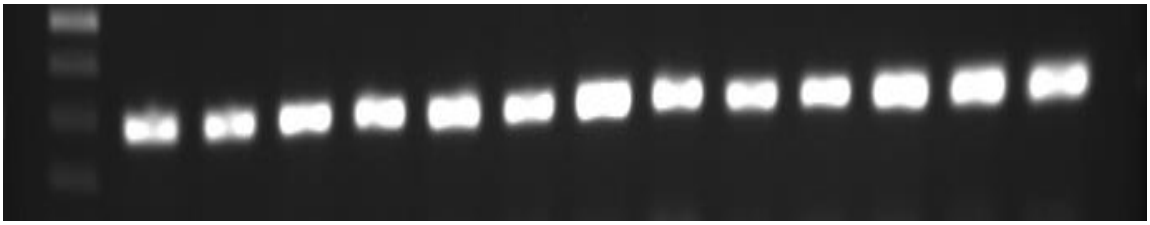


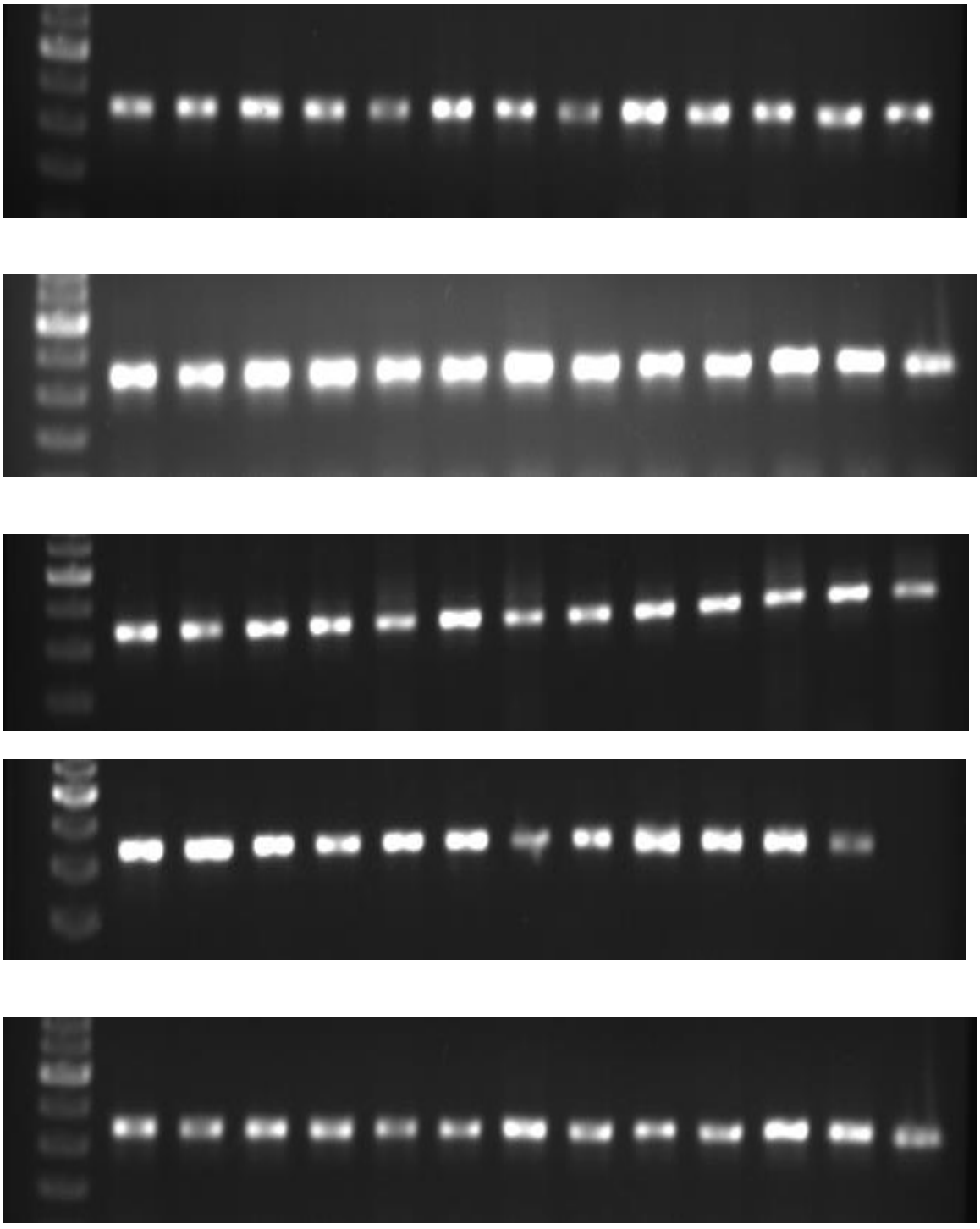


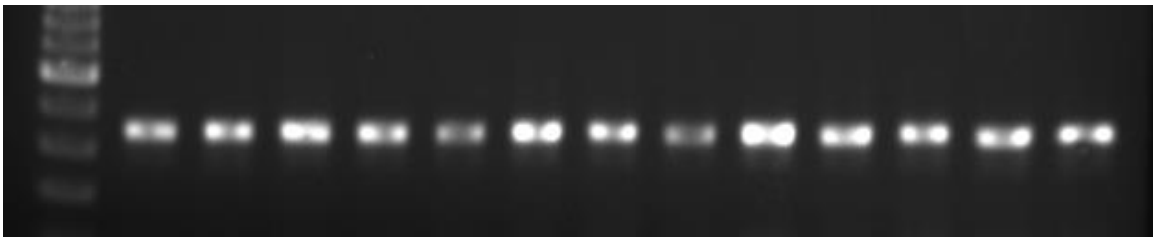


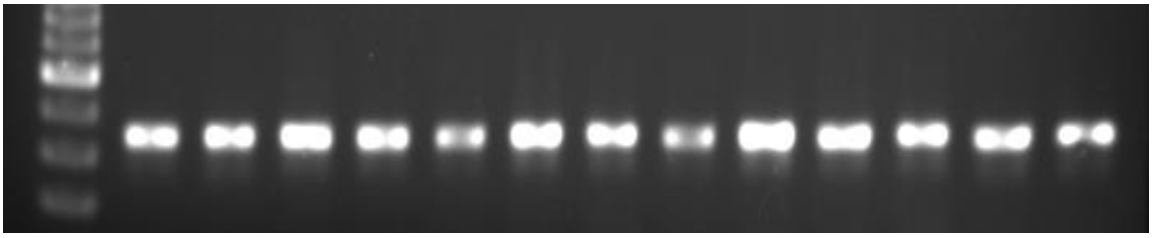


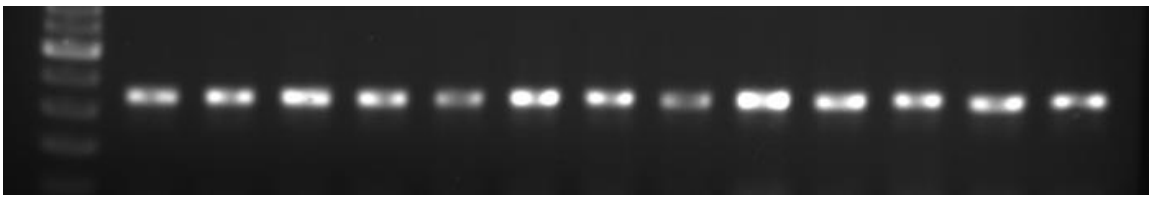


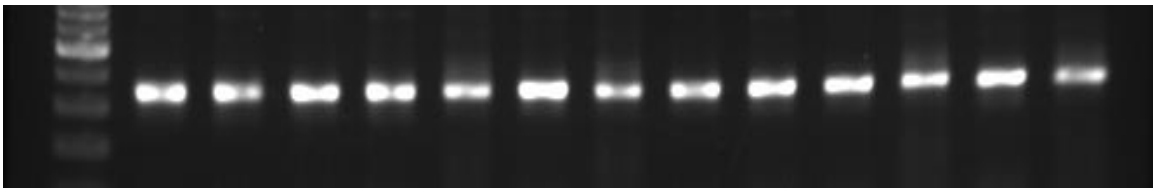


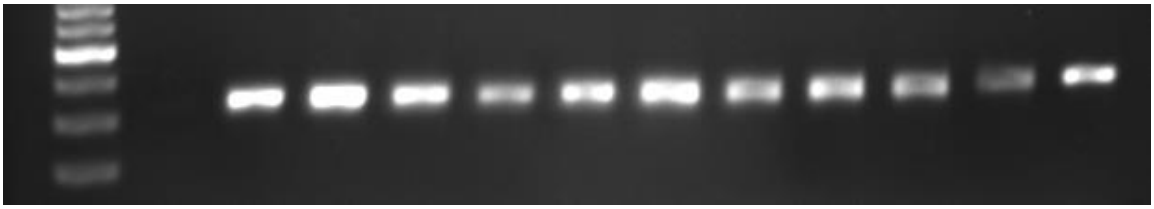


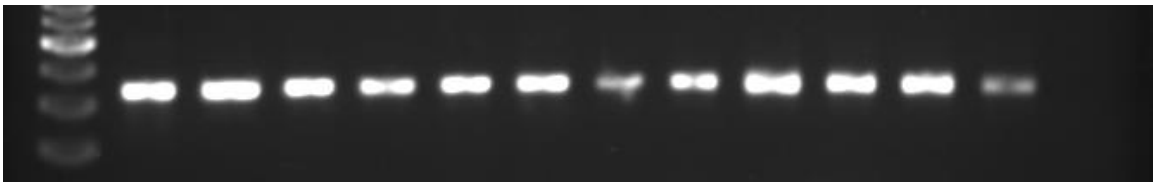


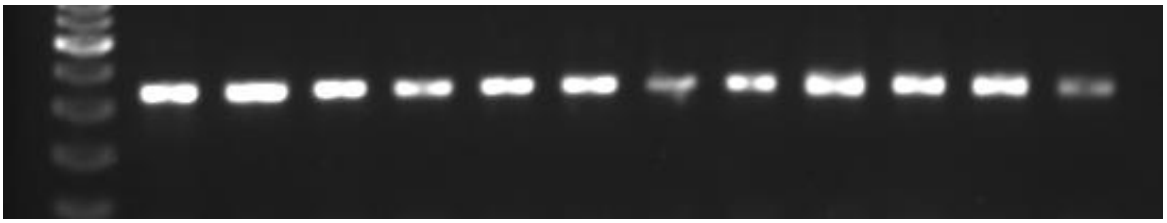


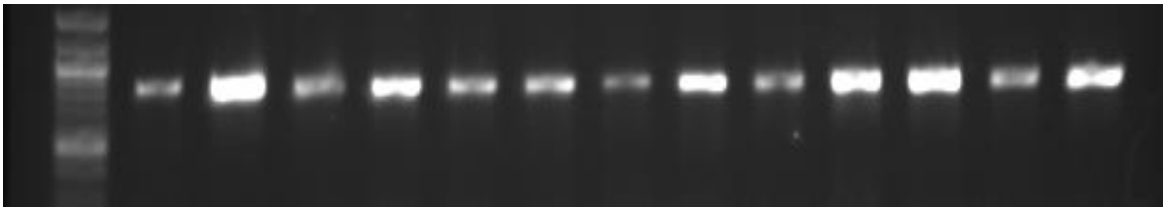


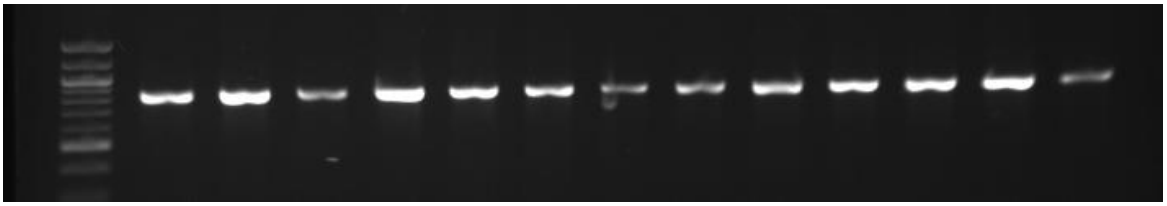


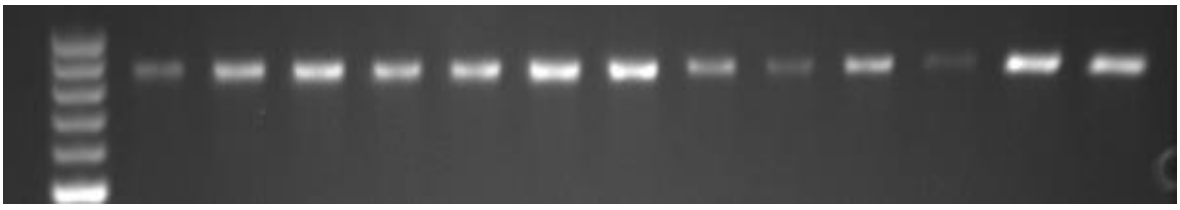


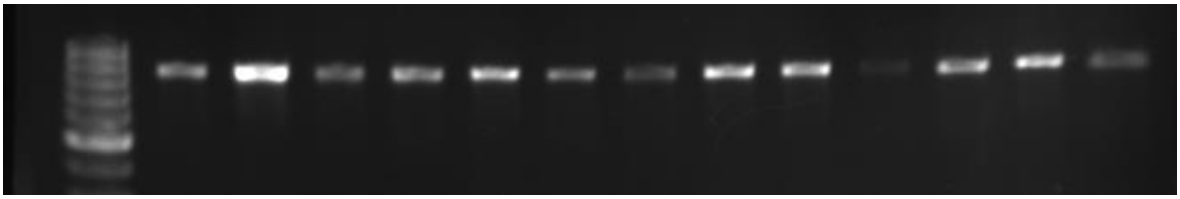


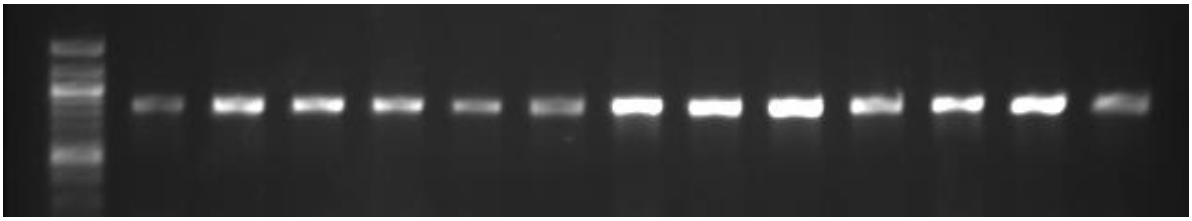


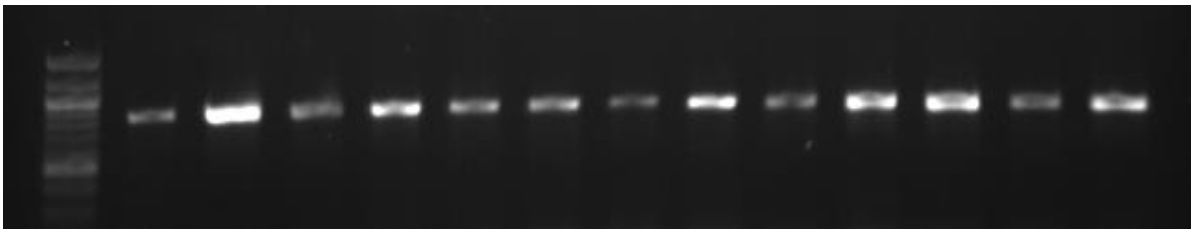


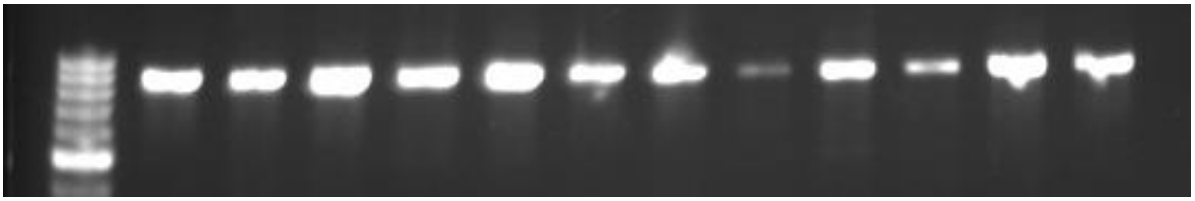


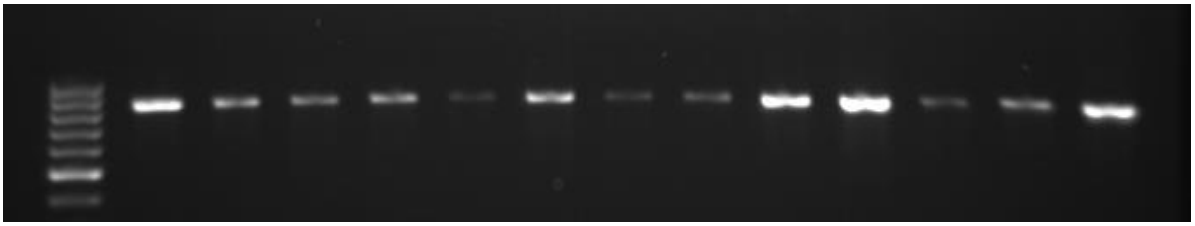


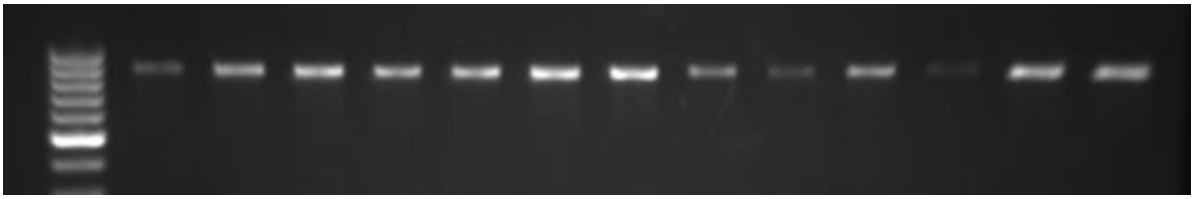


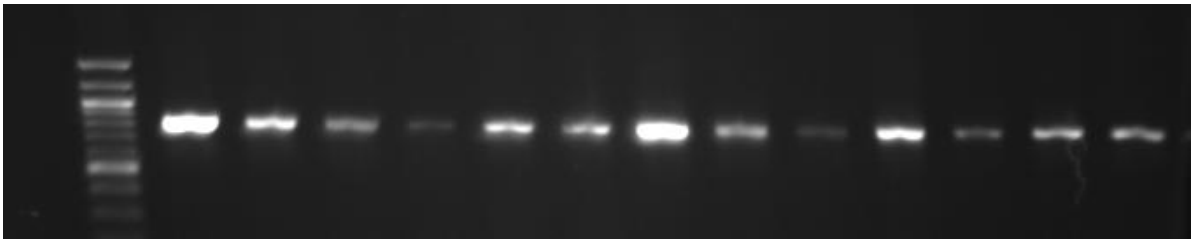


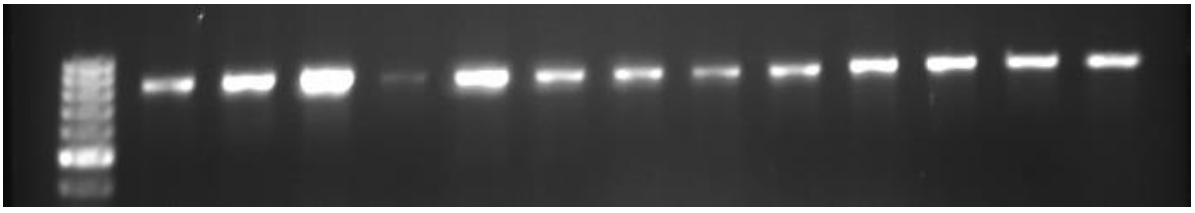


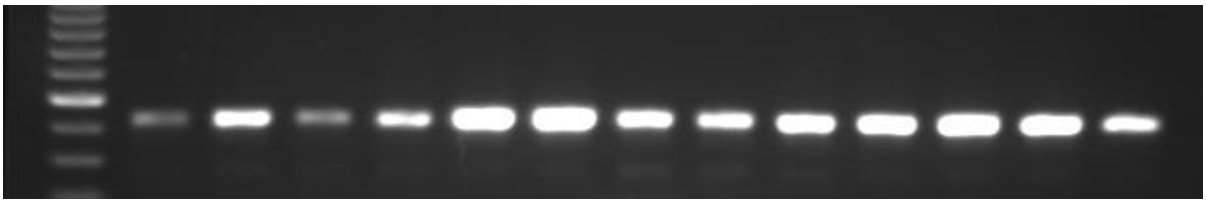


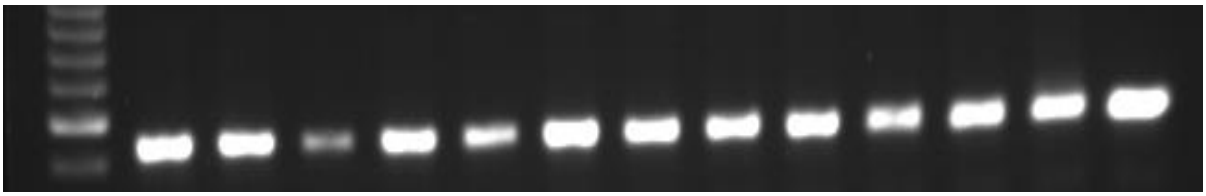


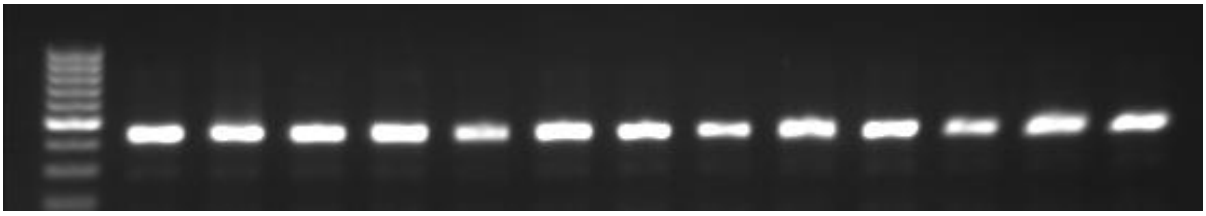


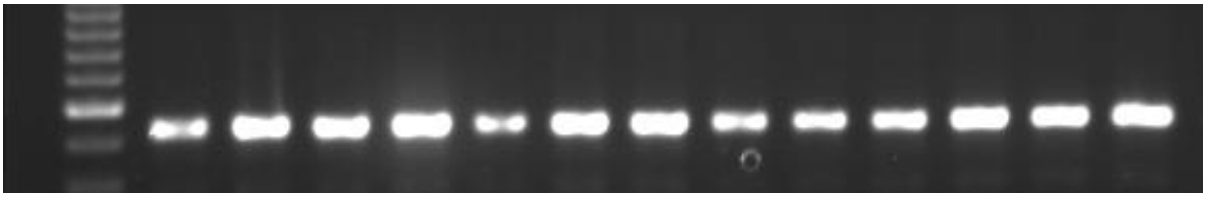


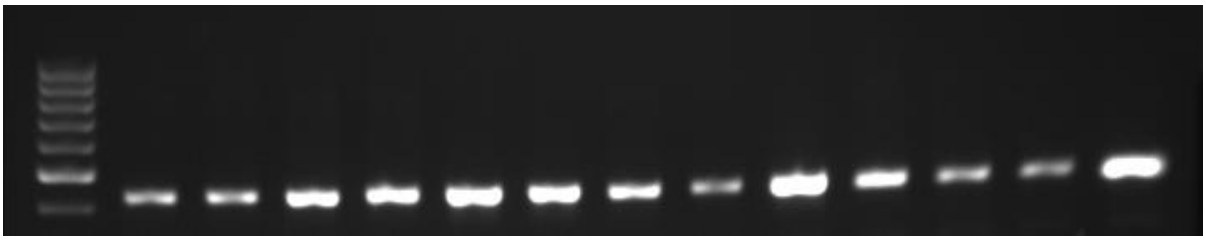


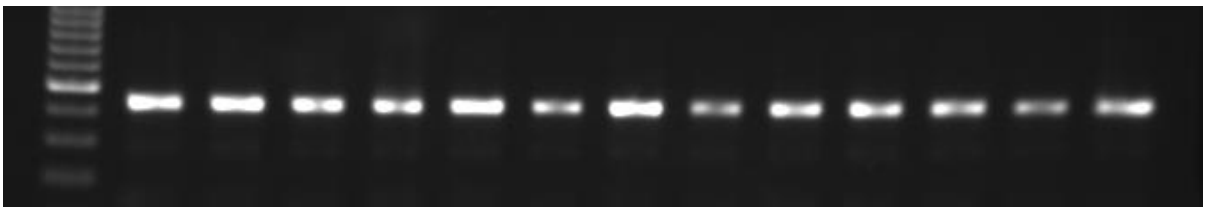


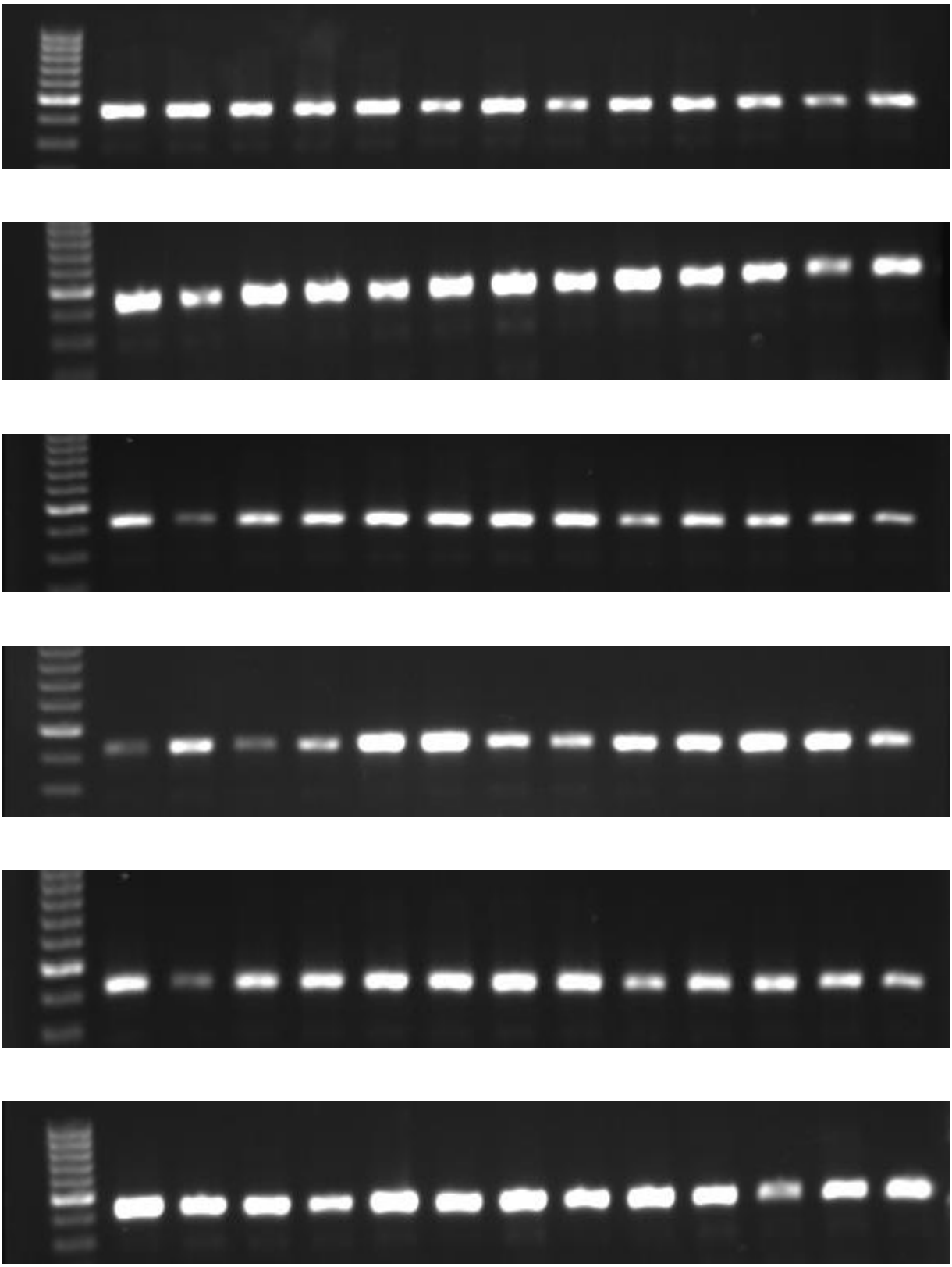






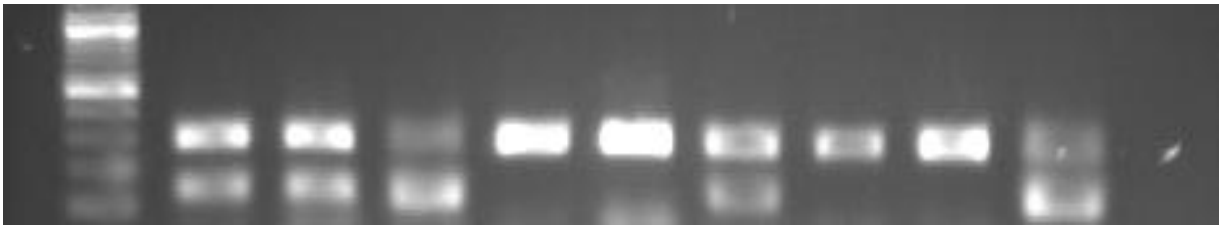


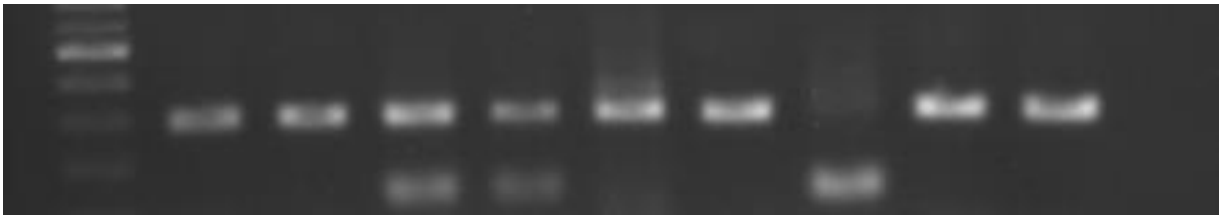


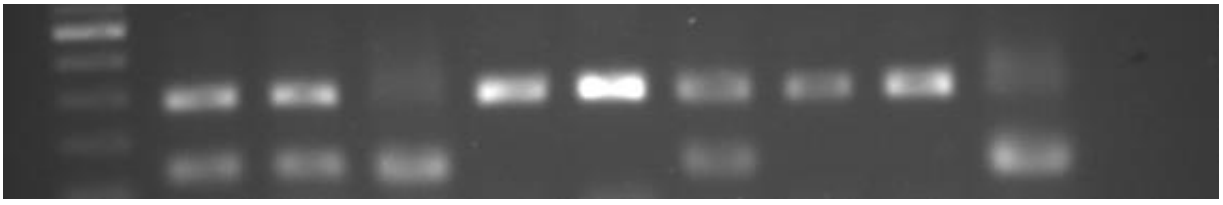


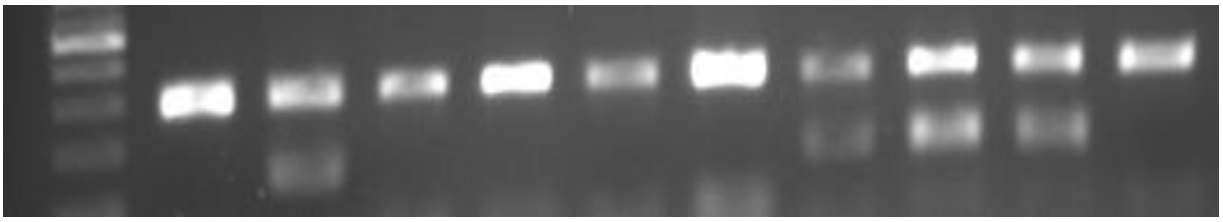
After the successful amplification of the selected genes restriction fragment length polymorphism (RFLP) analysis was performed in order to detect each individual genotype. Each PCR product was digested with the appropriate restriction enzyme (AvaII and XbaI respectively) at 37°C, as detailed in the methodological approach section. Following incubation, the digested products were visualized by agarose gel electrophoresis with ethidium bromide under UV

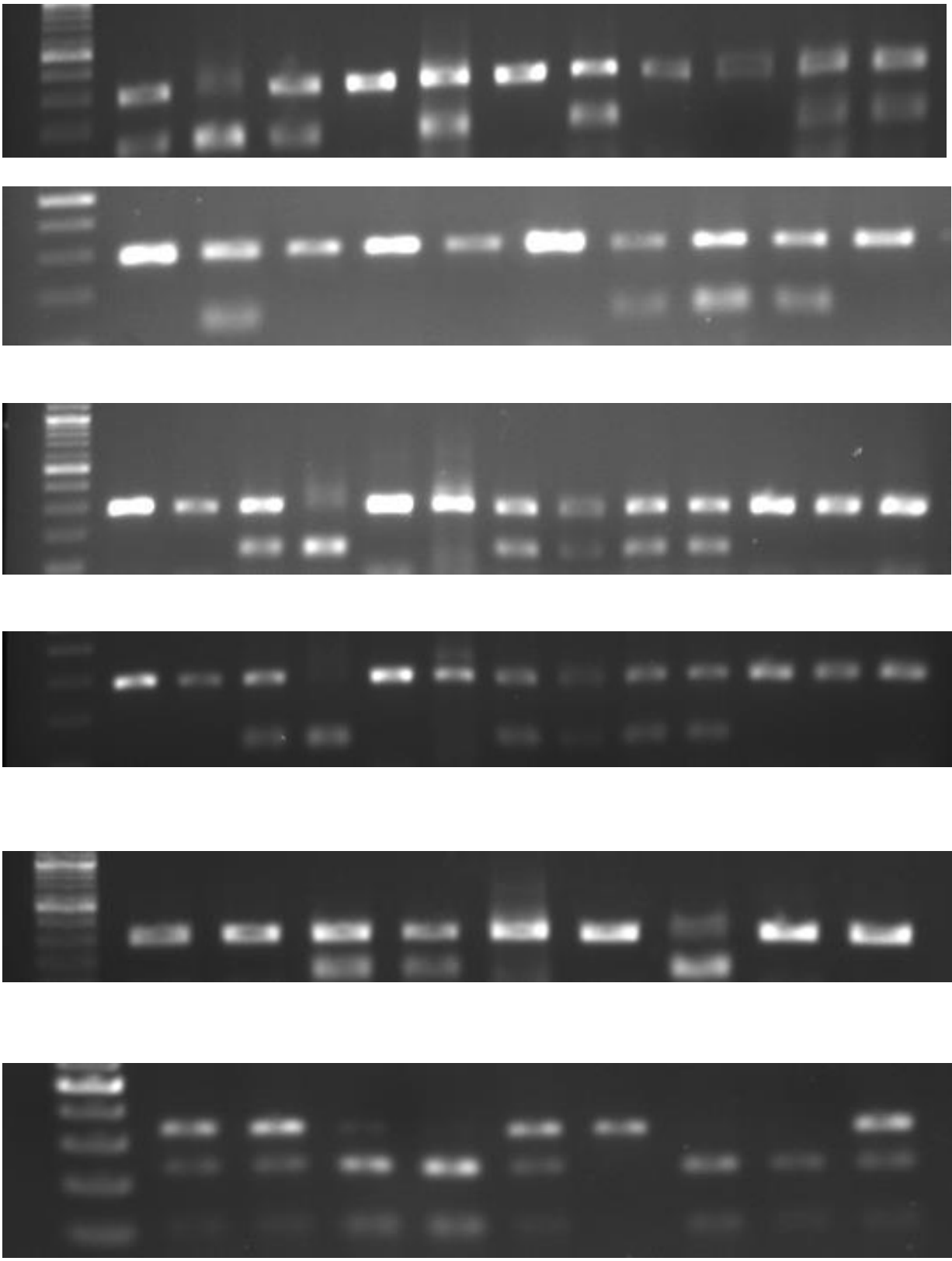
Below are indicative pictures of the RFLP-PCR products.

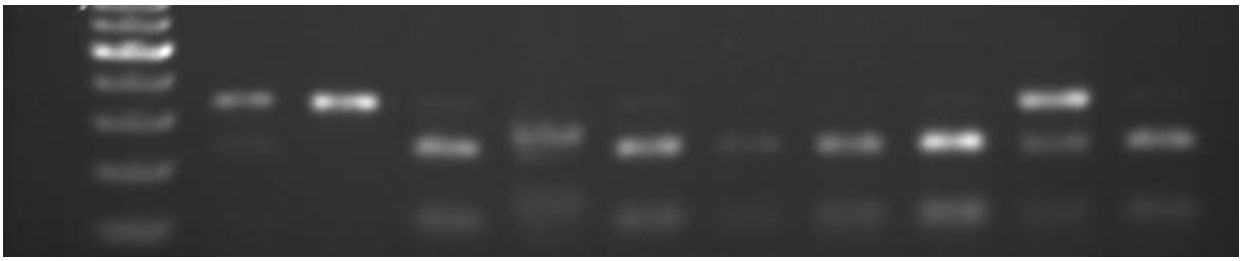


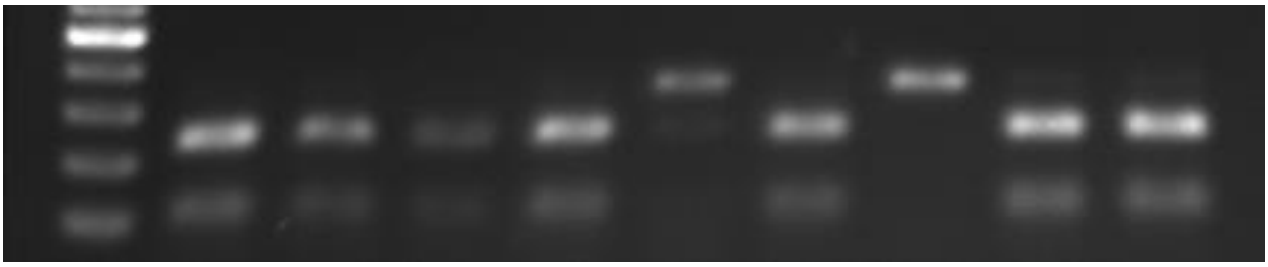


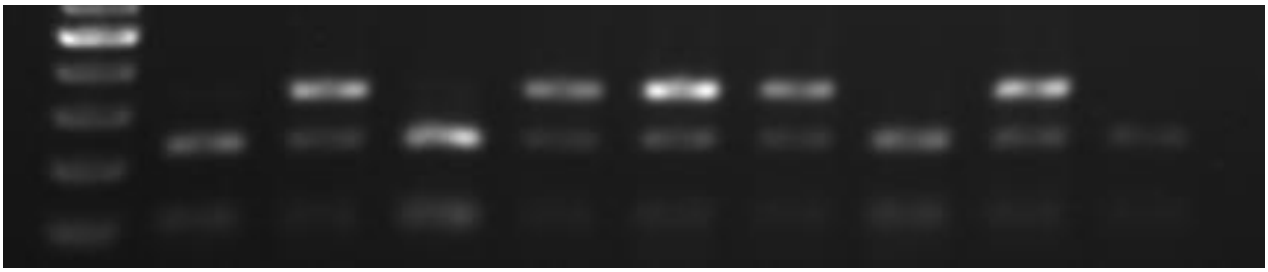


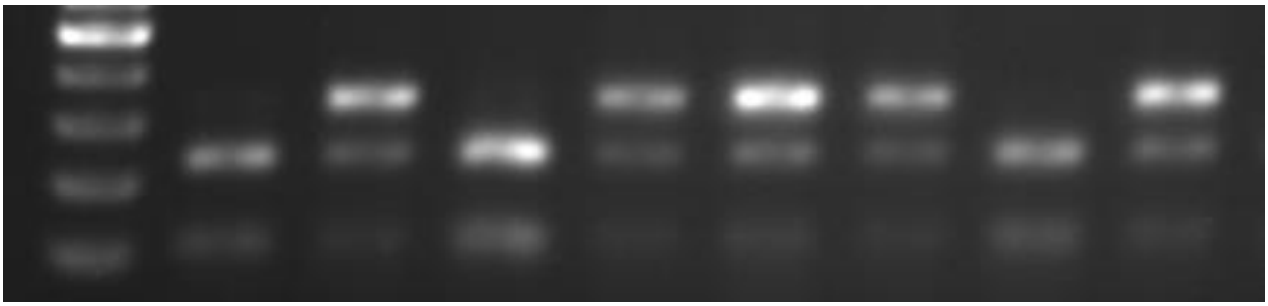


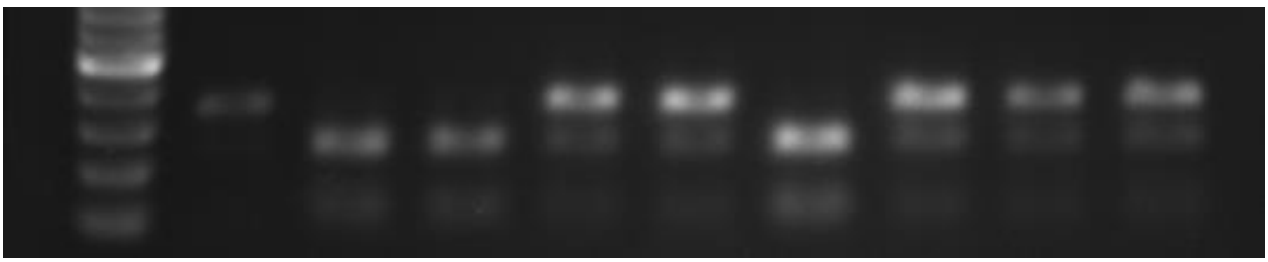


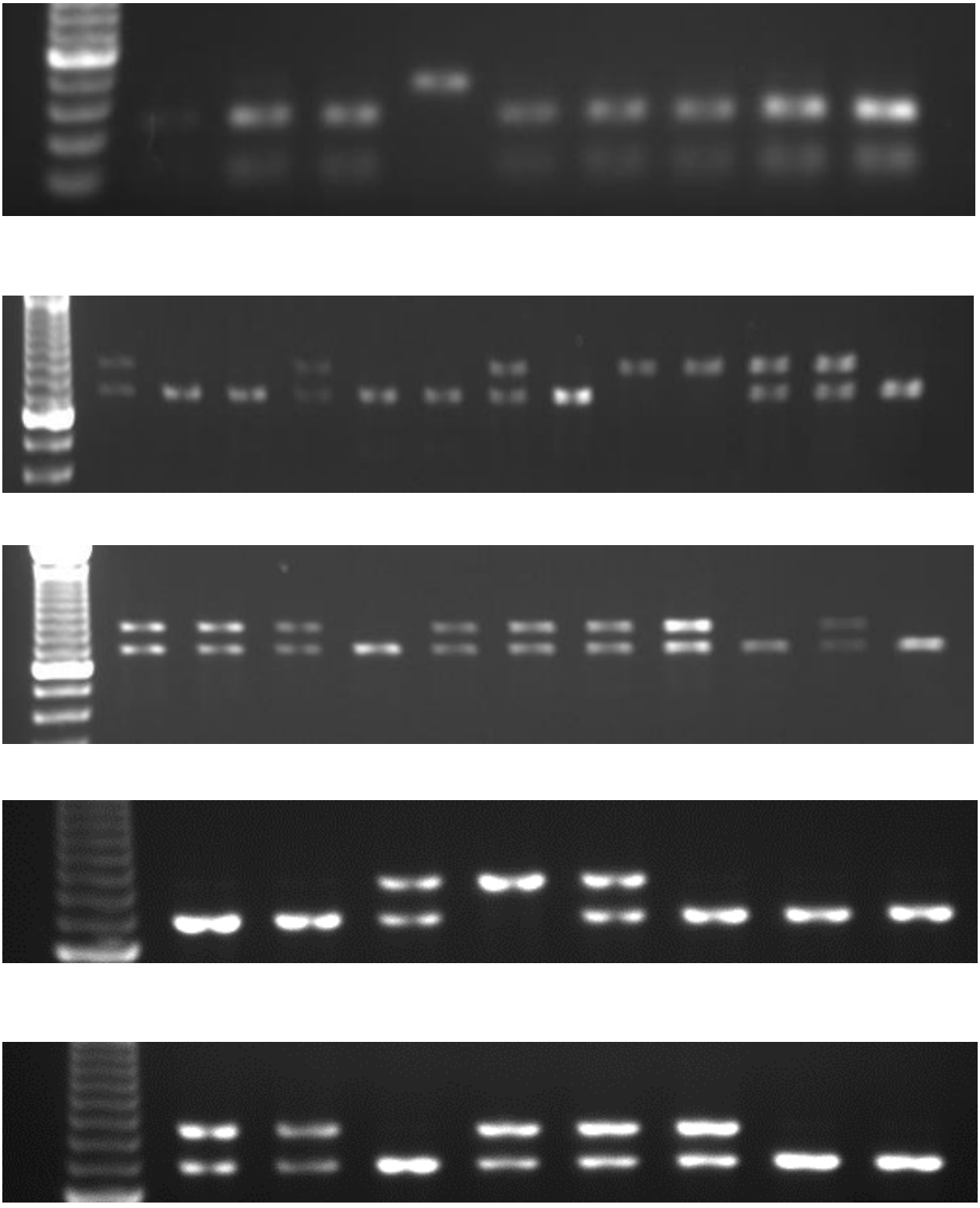


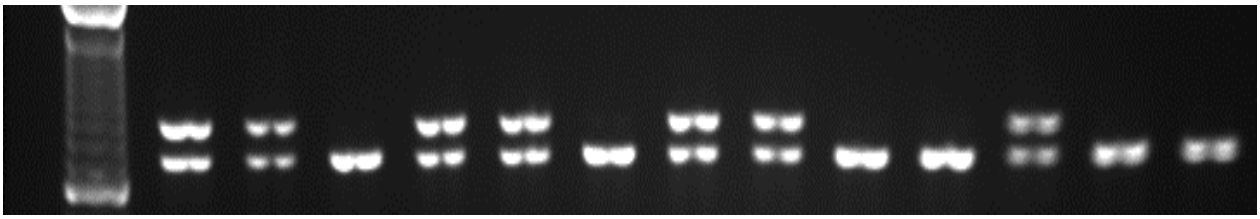


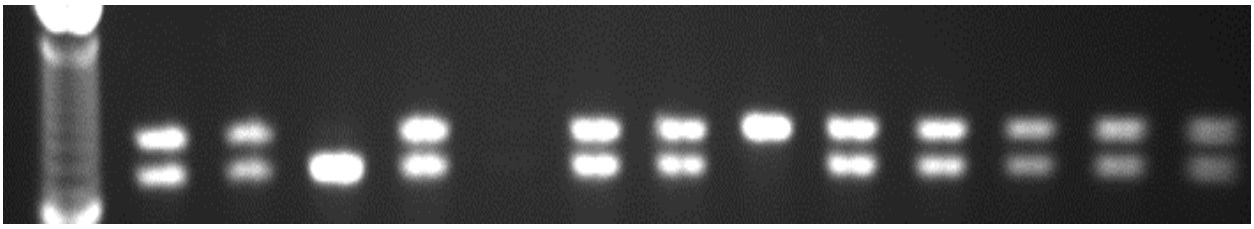


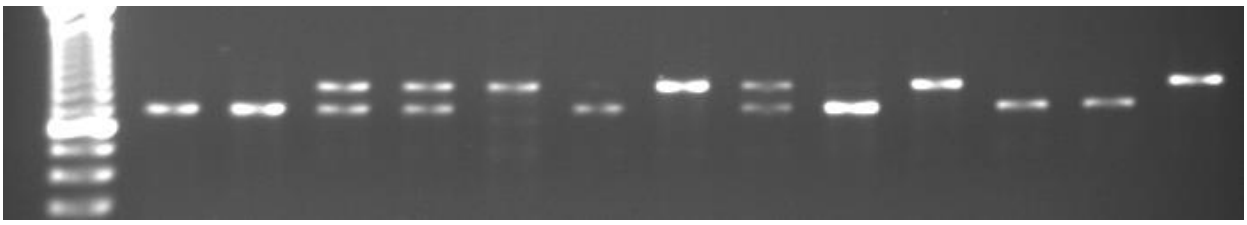


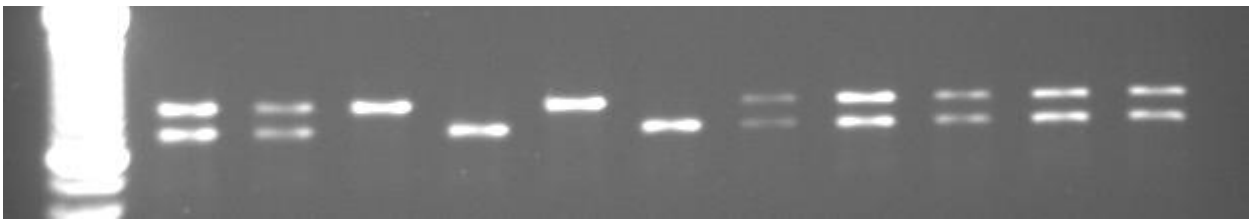


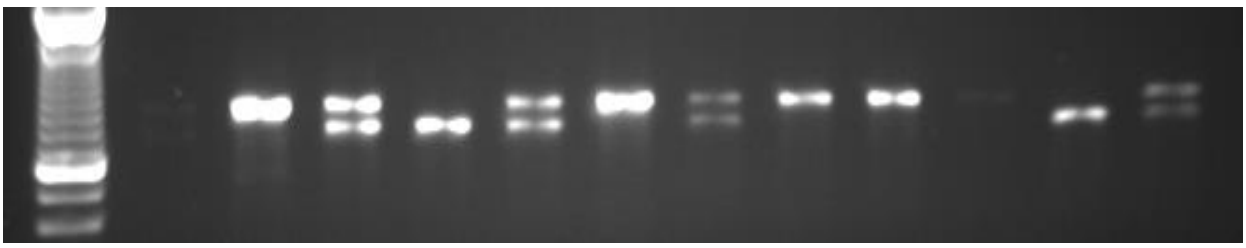


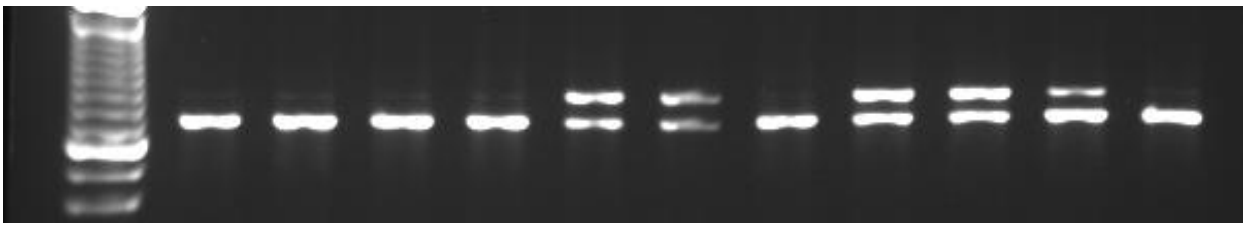


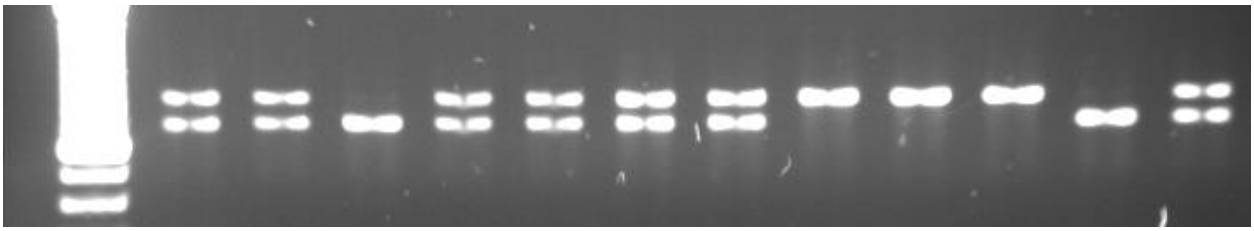


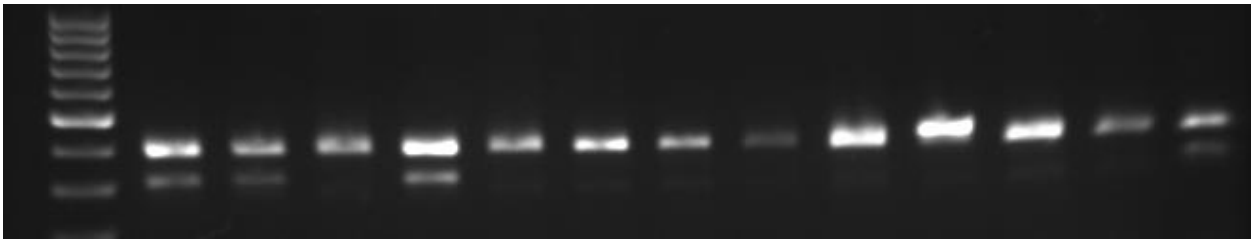


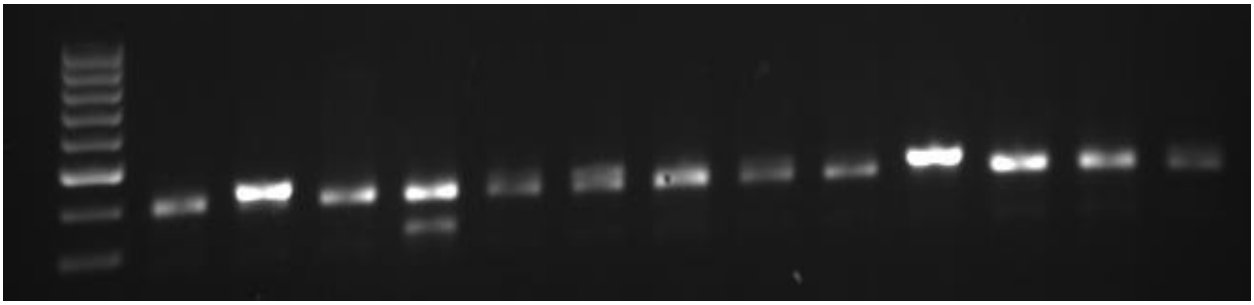


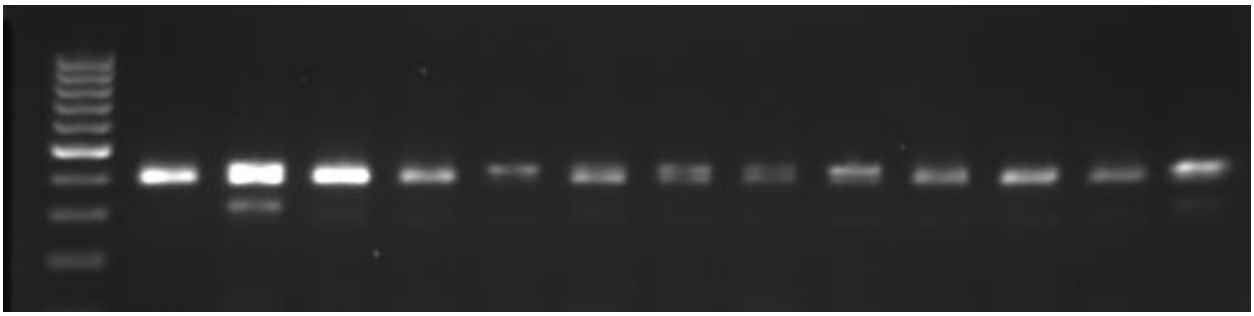


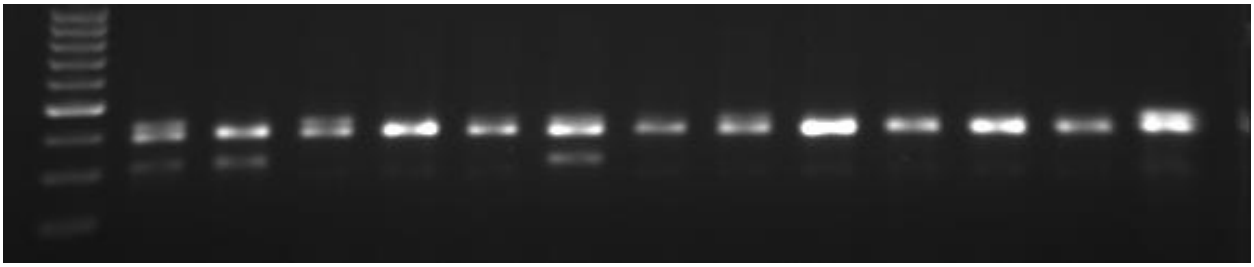


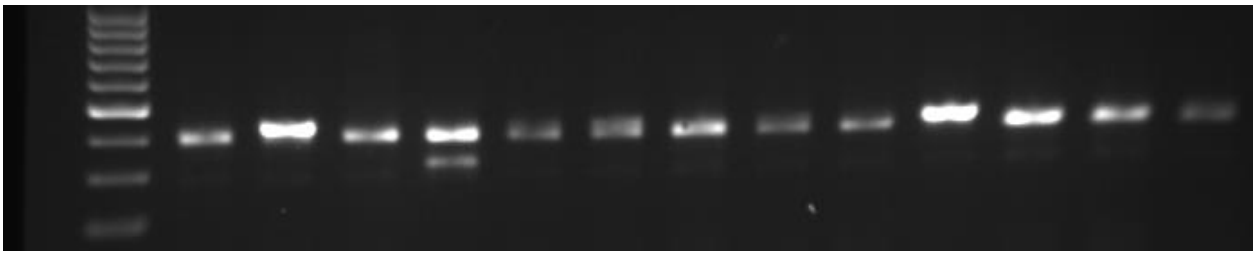


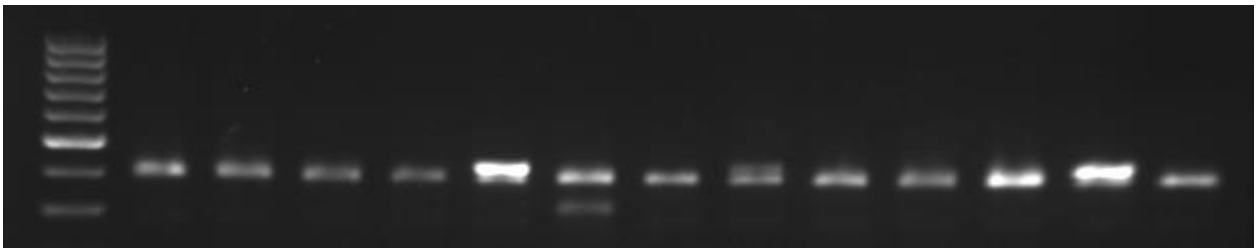


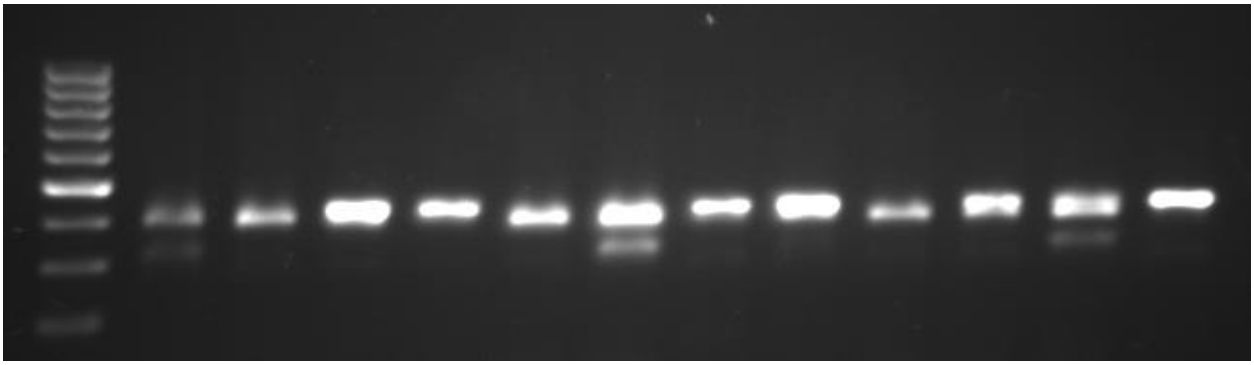


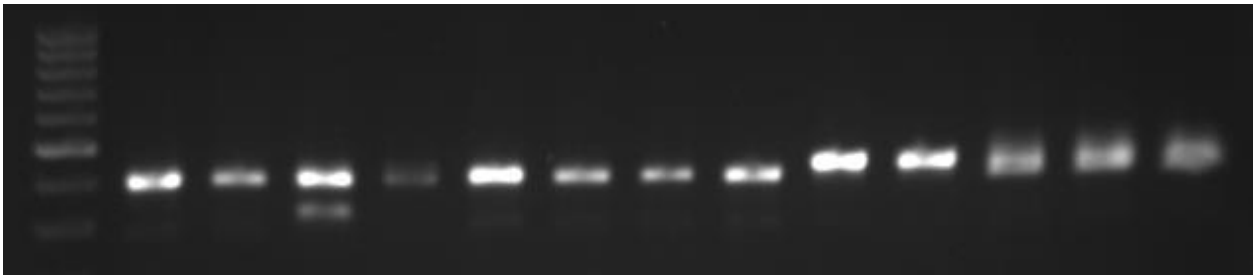












Following RFLP-PCR analysis the genotype of each individual ewe was detected and illustrated in the following tables.

POLYMORPHISM 1				
Genotypes	**++**	**+-**	**--**	**Total**
Number of ewes	167	62	31	260
Percentage %	64,2%	23,8%	12%	100%

Frequency and percentage of genotypes of polymorphism 1 in the investigated animals (n=260).

POLYMORPHISM 2				
Genotypes	**++**	**+-**	**--**	**Total**
Number of ewes	136	71	53	260
Percentage %	52,3%	27,3%	20,4%	100%

Frequency and percentage of genotypes of polymorphism 2 in the investigated animals (n=260).

The results presented in this study indicate that regarding polymorphism 1 the (++) genotype was present at 64,2%, following by 23,8% and 12% of the (+-) and (--) genotypes respectively.

Regarding the second polymorphism polymorphism , the (++) genotype was present at 52,3%, following by 27,3% and 20,4% of the (+-) and (--) genotypes respectively.

In livestock production, there is always a high interest in the improvement of the reproductive traits of animals. During the last years, improvement of the reproductive as well as other quantitative characteristics was based on selection programmes that take into consideration only the phenotype of the animals. However, this procedure is laborious, expensive and time consuming. In recent years, various Marker-assisted selection (MAS) programmes are used as complementary to the traditional selection methods in order to provide faster improvement in specific quantitative traits by associating a region of the DNA with a specific characteristic (Rothschild et al., 2000). Genetic improvement of reproductive traits in practical breeding programmes requires effective protocols for animal recording and summarisation and use of resulting records. In pedigree flocks, comprehensive recording and derivation of estimated breeding values can maximize rates of genetic improvement, but must be evaluated against anticipated returns from sales of superior breeding animals. In commercial flocks, where value per

animal marketed is lower than in pedigree flocks, benefits of detailed recording may best be captured by purchase of superior sires from progressive pedigree breeders. However, simple, cost-effective methods to identify replacement females from within the flock are still required.

Regarding sheep production, high prolificacy is one of the important goals of the sheep breeding in the world. The majority of sheep species produce single lamb and a small number will produce twin lambs, which greatly affected the breeding production. Owing to the low heritability of sheep lambing number (only from 0.03 to 0.1), it was difficult to improve this feature through traditional selection. Therefore, scientists pay more attention to search the candidate genes or mutations associated with ovulation rate and multiplets. As high prolificacy is a complex trait, and it is difficult to thoroughly identify the candidate genes related to this trait using the single molecular biology technique, which was affected by genes, age, season, and nutrition, and the genetic factor is the most important factor. Crossbreeding with prolific breeds or introgression of major genes for ovulation rate is recommended if a substantial increase is desired.

It is now well established that the use of major genes to increase prolificacy involves unique opportunities and challenges. Several mutations regarding ovulation rate can increase litter size by 0.5–1.0 lambs in heterozygotes and these mutations can be introgressed in different breed by repeated backcrossing and DNA testing, resulting in enhanced ovulation rates in an otherwise unmodified genetic background. The use of various mutations in genes is especially challenging, because of infertility in same cases in homozygous ewes and usually involves mating carrier males and females with DNA testing of offspring to identify the desired heterozygous replacement females. Thus, individual-animal identification (at least with regard to genotype) in addition to regular DNA genotyping and control of mating are required and may preclude effective use of various mutations in extensive or poorly controlled breeding programme, as reproductive ability has an important role in profitability of sheep production.

In different genes, several causative mutations with major effects on reproductive performance traits, such as ovulation rate and litter size have been identified in different sheep breeds around the world. The knowledge of the genes involved in ovulation rate and litter size and their effects provides useful information for breeding and selection on these traits. Moreover, the identification of the mutations affecting ovulation rate has provided new insights into the control of ovarian function (Juengel et al., 2013). Therefore, it is necessary to look for mutations with positive effects on prolificacy in different breeds and populations, around the world and this study performed such experiments in the Pelagonia sheep breed.

Characteristics of a major gene affecting prolificacy in a population include high variation in ovulation rate and litter size, combined with high repeatability. In multiple lines of

sheep single genes mutations that have major effects on ovulation rate have been identified. Major genes could be detected by genomic scanning and mapping of highly prolific sheep. A number of major prolificacy genes such as *BMPR1B* (bone morphogenetic protein receptor 1B), *BMP15* (bone morphogenetic protein 15), *GDF9* (growth differentiation factor 9) and *B4GALNT2* (beta-1,4-*N*-acetyl-galactosaminyl transferase 2), located on ovine chromosomes 6, X, 5 and 11, respectively, have been identified in sheep (Drouilhet et al., 2013; Galloway et al., 2000; Hanrahan et al., 2004; Souza et al., 2001). Selective breeding of sheep carrying these mutations can be used to increase prolificacy in the flock.

Individuals that carry two copies of the FecB allele are fertile, but increases in ovulation rates may exceed two, with corresponding increases in litter size greater than one. High frequencies of triplet or greater numbers of births in homozygous ewes may result in excessive neonatal deaths relative to non-carrier ewes and mandate specialized breeding programmes to generate heterozygous FecB ewes and exclude homozygous ewes from the breeding flock. The suitability of heterozygous ewes depends on the prolificacy of the recipient breeds. Thus, in the USA, prolificacy of adult ewes of common commercial breeds ranges from 1.75 to 1.95. Insertion of a single copy of FecB into this genetic background increases litter size by approximately one lamb, generally with unacceptable increases in frequency of large (>3) litters and with little or no increase in numbers of lambs weaned (Notter et al., 2008). In contrast, however, introgression of FecB into intensively managed commercial dairy flocks of Awassi and Assaf (an Awassi × East Friesian composite) ewes in Israel with baseline litter size of approximately 1.3 and 1.65, respectively, increased litter size to acceptable levels of 1.90 and 2.40, respectively, in heterozygotes and 1.92 and 2.55, respectively, in homozygotes (Gootwine, 2009).

In various sheep breeds, mutations that increase ovulation rate and affect reproductive performance have been discovered in the bone morphogenetic protein 15 gene (BMP15; breeds showing this mutation include the Inverdale, Hanna, Belclare, Cambridge, and Laucune; Shimasaki et al. 2003), its receptor gene (BMPR1B; the Booroola breed), and growth differentiation factor 9 gene (GDF9; the Cambridge and Belclare breeds) (Paradis et al. 2009). In Han sheep, point mutations of the BMP15 gene B2 (CfwdarwT), the BMPR1B gene FecB (A746G), and the GDF9 gene G3 (G477A) and one novel single nucleotide mutation (G729T) have been detected by PCR–SSCP or PCR–RFLP. Recent studies have also reported that incorporation of *FecB* gene in non-prolific sheep breeds can significantly enhance their reproductive performance. This is because one copy of the gene has a potential to increase ovulation rate by about 1.5 and two copies by about 3.0. Indeed, during the last years the *FecB* gene has been introgressed from the Booroola Merino sheep breed into several other breeds in at least 13 different countries in order to improve the reproduction rate while maintaining desirable levels of performance for other traits. High-accuracy marker tests have been developed for the Booroola mutation

(Walkden-Brown et al., 2008), and this mutation is reported in Indian sheep breeds (Garole and Kendrapada) (Fogarty, 2009), Chinese sheep breeds, Small Tail Han and Hu (Chu et al., 2007) and Javanese sheep (Davis et al., 2006) an Indonesian breed. Cross-breeding and introgressive hybridization of the Booroola mutation in Indian, Chinese, French and Israeli sheep populations were successful. However, introduction of the Booroola mutation was not successful in Australia and USA (Mulsant et al., 2003; Walkden-Brown et al., 2008). Recently, two new mutations in BMP15 have been described in the French Grivette and the Polish Olkuska breeds. In addition the *FecXI* gene has now been introgressed into many different sheep breeds to take advantage of the increased ovulation and lambing rates in the heterozygous female carriers. Previous studies have also reported that high prolificacy in Booroola sheep is the result of a mutation (*FecB*) in the bone morphogenetic protein receptor IB (*BMPRIB*) gene (Wilson et al., 2001; Mulsant et al., 2001; Souza et al., 2001). In *FecBB* animals, a single A to G substitution at nucleotide position 830 results in an arginine replacing a glutamine amino acid in a highly conserved region of this receptor. Knowledge of *FecB* gene has prompted researchers to screen other breeds of sheep to determine whether the gene is responsible for their high prolificacies. Furthermore, other studies have reported that high prolificacy in sheep carrying the Booroola gene (FecB) is the result of a mutation in the BMPIB receptor and high prolificacy in Inverdale sheep (FecXI) is the result of a mutation in the BMP15 oocyte-derived growth factor gene have allowed direct marker tests to be developed for FecB and FecXI. These tests were carried out in seven strains of sheep (Javanese, Thoka, Woodlands, Olkuska, Lacaune, Belclare, and Cambridge) in which inheritance patterns have suggested the presence of major genes affecting prolificacy and in the prolific Garole sheep of India, which have been proposed as the ancestor of Australian Booroola Merinos. The FecB mutation was found in the Garole and Javanese sheep but not in Thoka,Woodlands, Olkuska, Lacaune, Belclare, and Cambridge sheep. The *FecB* gene was present in some Chinese prolific breeds of sheep, such as Huyang, Small Tail Han (STH), Cele, Duolang sheep and Chinese Merino prolific strains, but absent in the low prolific sheep breeds such as Mongolia, Chinese Merino, Tan, Xinjiang, Hulunbeier, Inner Mongolia FineWool and Northeastern Half-fuzz Sheep. It has been confirmed that *FecB* gene was associated with high prolificacy in some Chinese breeds or strains of sheep. Use of FecB in low-input smallholder flocks in India permitted a rapid increase in prolificacy in lowly prolific Deccani ewes (mean litter size of 1.03 (Nimbkar et al., 2007, 2008). In that study, heterozygous ewes had average litter size of approximately 1.5 live lambs at birth and average litter size for a small number of homozygous ewes was 1.65. When coupled with outreach and training activities to achieve incremental improvements in management and nutrition appropriate to this level of prolificacy, weight of lamb produced and gross margin per breeding ewe increased by 7.5% and 37–50%, respectively, for heterozygous compared to wild type ewes. Also, in Deccani ewes, the

impact of a second copy of FecB was much smaller than that of a single copy, suggesting that avoidance of homozygous ewes may be less important in programmes involving lowly prolific ewes.

An investigation on British and Irish sheep breeds showed that the Lleyn breed (a breed of sheep from the Lleyn peninsula, in Gwynedd, north-west Wales) is the most likely source of *FecXG* mutation in Belclare and Cambridge sheep. The *FecXB* mutation came from a fertile line, developed using prolific ewes of commercial flocks in Ireland in the 1960s and then used in the genesis of the Belclare breed (Mullen et al., 2013). In fact, *BMP15* gene could be considered as the most polymorphic locus among the genes affecting prolificacy in sheep. A new mutation in exon 2 of *BMP15* associated with an increase in prolificacy was also reported in two Iranian sheep breeds. Moreover, introducing *FecB* gene to some low prolific breeds of sheep by crossbreeding system can improve the reproductive traits.

The results present in this study, regarding the genotyping of Pelagonia sheep breed, suggest that continuous screening and crossbreeding system can be used in order to improve the reproductive traits of this breed when desired.

Conclusions

A major step in order to improve the economic efficiency in the sheep industry is to improve the reproductive performance of a breed.

As DNA tests can now be performed for both the *FecB* and *FecXI* genes, and with the expectation that more DNA tests will soon be developed for other prolificacy genes, in future there will be more extensive use of major genes for prolificacy in many different breeds through both natural and artificial breeding programmes.

Performing DNA tests in rams of different breeds, which carry major genes for prolificacy, will allow breeders to choose the desired size of effect within a breed that is best adapted to their management conditions.

The status of the *FecB* and *FecX^{I}* gene mutations were studied in ewes from the Pelagonia sheep breed. In view of our results, as gene technology has been used successfully to utilize fecundity genes such as *FecB* and *FecX^{I}* in many countries around the world, incorporation of these genes in Greek sheep breeds can increase litter size of these local breeds and will be of considerable economic value to Greek sheep producers. As DNA tests are the key to the utilisation of these genes in the sheep industry, the PCR-RFLP tests performed in this study were found to very useful tools for future breeding plans in this sheep breeds. The DNA mutation tests for both genes, performed in this study, will enable breeding plans to be developed that allow the most effective use of these genes in Pelagonia sheep breed.

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