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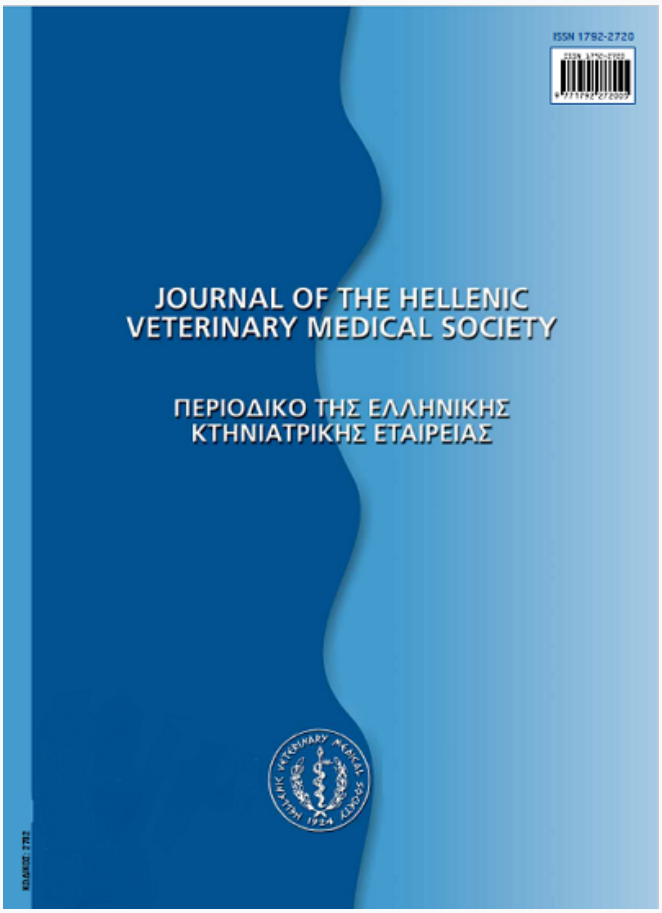


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*I. MASTRANESTASIS (Ι. ΜΑΣΤΡΑΝΕΣΤΑΣΗΣ), Ch. LIGDA (Χρ. ΛΙΓΔΑ), K. THEODOROU (Κ. ΘΕΟΔΩΡΟΥ), L. V. EKATERINIADOU (Λ.Β. ΑΙΚΑΤΕΡΙΝΙΑΔΟΥ)*

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# Genetic structure and diversity among three Greek sheep breeds using Random Amplified Polymorphic DNA-PCR

**Mastranestasis I.,** MSc[a,b], **Ligda Ch.,** PhD[b], **Theodorou K.,** Eng., MSc, PhD[a], **Ekateriniadou L.V.,** MSc., PhD[b]

[a] *Biodiversity Conservation Laboratory, Department of Environment, University of the Aegean, 811 00 Mytilene, Greece.*

[b] *National Agriculture Research Foundation, - NAGREF, Veterinary Research Institute of Thessaloniki, NAGREF Campus of Thermi, P.O.Box 60272, 570 01 Thermi, Greece*

# Γενετική δομή και ποικιλότητα των φυλών προβάτου Λέσβου, Χίου και Καραγκούνικης με τη χρήση τυχαίως ενισχυμένων πολυμορφικών τμημάτων DNA (RAPD-DNA)

**Ι. Μαστρανεστάσης,** MSc[a,b], **Χρ. Λίγδα,** PhD,[b], **Κ. Θεοδώρου,** Eng., MSc, PhD[a], **Λ. Β. Αικατερινιάδου,** MSc., PhD[b]

[a] *Πανεπιστήμιο Αιγαίου, Τμήμα Περιβάλλοντος, Εργαστήριο Διατήρησης Βιοποικιλότητας, Λόφος Πανεπιστημίου, 811 00 Μυτιλήνη*

[b] *ΕΘνικό Ίδρυμα Αγροτικής Έρευνας, Ινστιτούτο Κτηνιατρικών Ερευνών Θεσσαλονίκης, Κτήμα Θέρμης ΕΘΙΑΓΕ, Τ.Θ. 60272, 570 01 Θέρμη, Θεσσαλονίκη*

**ABSTRACT.** Genetic structure and diversity of 120 animals from three Greek local breeds were investigated by Random Amplified Polymorphic DNA (RAPD) - PCR method. Sheep samples originated from the Lesvos, Chios and Karagouniko breeds were treated with 11 random primers to estimate their genetic diversity and phylogenetic relationships. Our analysis comprised two levels of the breeds' genetic structure: i) the genetic differentiation among the three breeds and ii) the genetic differentiation among the flocks within each breed. This combined approach gave two main findings: i) the study of genetic distances and identity revealed that the Karagouniko sheep breed is genetically distinct from Chios (GD=0.1979) and Lesvos (GD=0.1691) breeds, while ii) the Chios and Lesvos breeds are genetically similar (GI=0.9631); half of the flocks of Lesvos have a relatively closer relationship with those of Chios than with the other Lesvos flocks. This is the first study that reports the close genetic relationship between the Chios and Lesvos breeds and gives strong evidence to hypotheses about their related origin. Furthermore, the study of polymorphic loci revealed particular indicators located in Karagouniko breed, as definitional datum of genetic identity or as a fingerprint of breed. Therefore, RAPD-DNA methods can be an efficient tool for the determination of phylogenetic relationships and genetic identity among the bloodstock breed of sheep.

***Keywords:*** Lesvos, Chios, Karagouniko sheep breeds, genetic structure, genetic diversity, RAPD-DNA

*Correspondence:* Viron-Ekateriniadou L.
National Agriculture Research Foundation, - NAGREF, Veterinary Research Institute of Thessaloniki, NAGREF Campus of Thermi, P.O. Box 60272, 570 01 Thermi, Greece
Tel.: +30 2310 365392, Fax: +30 2310 365371, E-mail: ekateriniadou@vri.gr

*Αλληλογραφία:* Λ. Βύρωνα-Αικατερινιάδου
ΕΘνικό Ίδρυμα Αγροτικής Έρευνας, Ινστιτούτο Κτηνιατρικών Ερευνών Θεσσαλονίκης, Κτήμα Θέρμης ΕΘΙΑΓΕ, Τ.Θ. 60272, 570 01 Θέρμη, Θεσσαλονίκη
Τηλ.: 2310 365392, Fax: 2310 365371, E-mail: ekateriniadou@vri.gr

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**ΠΕΡΙΛΗΨΗ.** Σκοπός της εργασίας είναι η διερεύνηση της γενετικής δομής και της ποικιλότητας των ελληνικών φυλών προβάτων Λέσβου, Καραγκούνικης και Χίου. Η μέθοδος ανάλυσης DNA που χρησιμοποιήθηκε ήταν η αλυσιδωτή αντίδραση της πολυμεράσης με τυχαίως ενισχυμένα πολυμορφικά τμήματα DNA (PCR - RAPD). Αναλύθηκαν συνολικά 120 μη συγγενή άτομα (40 ανά φυλή) με τη χρήση 11 ολιγονουκλεοτιδικών εκκινητών. Το υλικό προέρχεται από 22 εκτροφές, δέκα ποίμνια από τη Λέσβο, τρία ποίμνια από τη Χίο και ένα από το Σταθμό Γεωργικής Έρευνας της Χαλκιδικής, για τις φυλές Λέσβου και Χίου, αντίστοιχα. Επίσης, οκτώ ποίμνια της Καραγκούνικης φυλής από τους Νομούς Τρικάλων (3) και Καρδίτσας (5). Στη φυλή Καραγκούνικη, πολυμορφικές ζώνες εμφανίστηκαν σε όλους του εκκινητές, σε αντίθεση με τις φυλές Λέσβου και Χίου όπου ένας εκκινητής δεν εμφάνισε πολυμορφικές ζώνες. Οι πολυμορφικές ζώνες κυμαίνονταν από 0 έως 9 ανάλογα με το ζεύγος των εκκινητών και τη φυλή. Η φυλή Λέσβου εμφάνισε το μεγαλύτερο αριθμό πολυμορφικών ζωνών, ιδιαίτερα στους εκκινητές ALI 17, OPA 02 και OPF 05. Ο συνολικός αριθμός των πολυμορφικών ζωνών ήταν 61 στη φυλή Λέσβου, 57 στη φυλή Χίου και 39 στη Καραγκούνικη φυλή. Από τον υπολογισμό των φυλογενετικών σχέσεων προέκυψαν δυο κύρια αποτελέσματα: i) η γενετική απόσταση της Καραγκούνικης φυλής ήταν διακριτή από τη φυλή Χίου (GD=0.1979) και Λέσβου (GD=0.1691), ενώ ii) οι φυλές Χίου και Λέσβου εμφάνισαν σχετικά κλειστή φυλογενετική σχέση με γενετική ομοιότητα GI=0.9631. Τα μισά κοπάδια της φυλής Λέσβου παρουσίασαν σχετικά στενότερη φυλογενετική σχέση με τα κοπάδια της φυλής Χίου. Επίσης, από τη μελέτη των πολυμορφικών ζωνών, βρέθηκε μοριακός δείκτης που εμφανίζεται μόνο στα άτομα της Καραγκούνικης φυλής: ο εκκινητής ALI 01 εμφάνισε 4 πολυμορφικές ζώνες οι οποίες φαίνεται να χαρακτηρίζουν όλα τα άτομα της φυλής, ενώ δεν εμφανίζονται σε κανένα από τα άτομα των άλλων δύο φυλών. Ο φυλετικός αυτός δείκτης παρουσιάζει ενδιαφέρον για περαιτέρω έρευνα. Η συνολική παραλλακτικότητα (Ht) ανάμεσα στα κοπάδια ανά φυλή ήταν 0.2163 ± 0.0317, 0.1295 ± 0.0273 και 0.2138 ± 0.0410 για τη Λέσβου, Χίου και Καραγκούνικη, αντίστοιχα, ενώ η μέση παραλλακτικότητα εντός των κοπαδιών (Hs) ήταν 0.1549 ± 0.0161, 0.0912 ± 0.0145 και 0.1847 ± 0.0313 για τη φυλή Λέσβου, Καραγκούνικη και Χίου, αντίστοιχα. Η μέση γενετική διαφοροποίηση (Gst) μεταξύ των κοπαδιών υπολογίστηκε σε 0.2840 (Λέσβου), 0.2961 (Καραγκούνικο) και 0.1361 (Χίου). Για τον υπολογισμό των γενετικών αποστάσεων μεταξύ και εντός των φυλών χρησιμοποιήθηκε το λογισμικό πρόγραμμα POPGENE 1.31. Τα αποτελέσματα από την ανάλυση της γενετικής ποικιλότητας με τη χρήση των μοριακών δεικτών, σε συνδυασμό με τα γενεαλογικά δεδομένα, μπορούν να αξιοποιηθούν στα προγράμματα γενετικής βελτίωσης και διατήρησης των φυλών προβάτων. Χρειάζεται περαίτερω έρευνα για τη σύνδεση της γενετικής και φαινοτυπικής παραλλακτικότητας, με παραμέτρους του περιβάλλοντος παραγωγής (φυσικό και διαχείρισης), με στόχο τον προσδιορισμό των ιδιοτήτων προσαρμογής και την ανάπτυξη κατάλληλων στρατηγικών διαχείρισης.

***Λέξεις ευρετηρίασης:*** Φυλή Λέσβου, Φυλή Χίου, Φυλή Καραγκούνικη, γενετική δομή, γενετική ποικιλότητα, τυχαία ενισχυμένα πολυμορφικά τμήματα (RAPD-DNA)

## INTRODUCTION

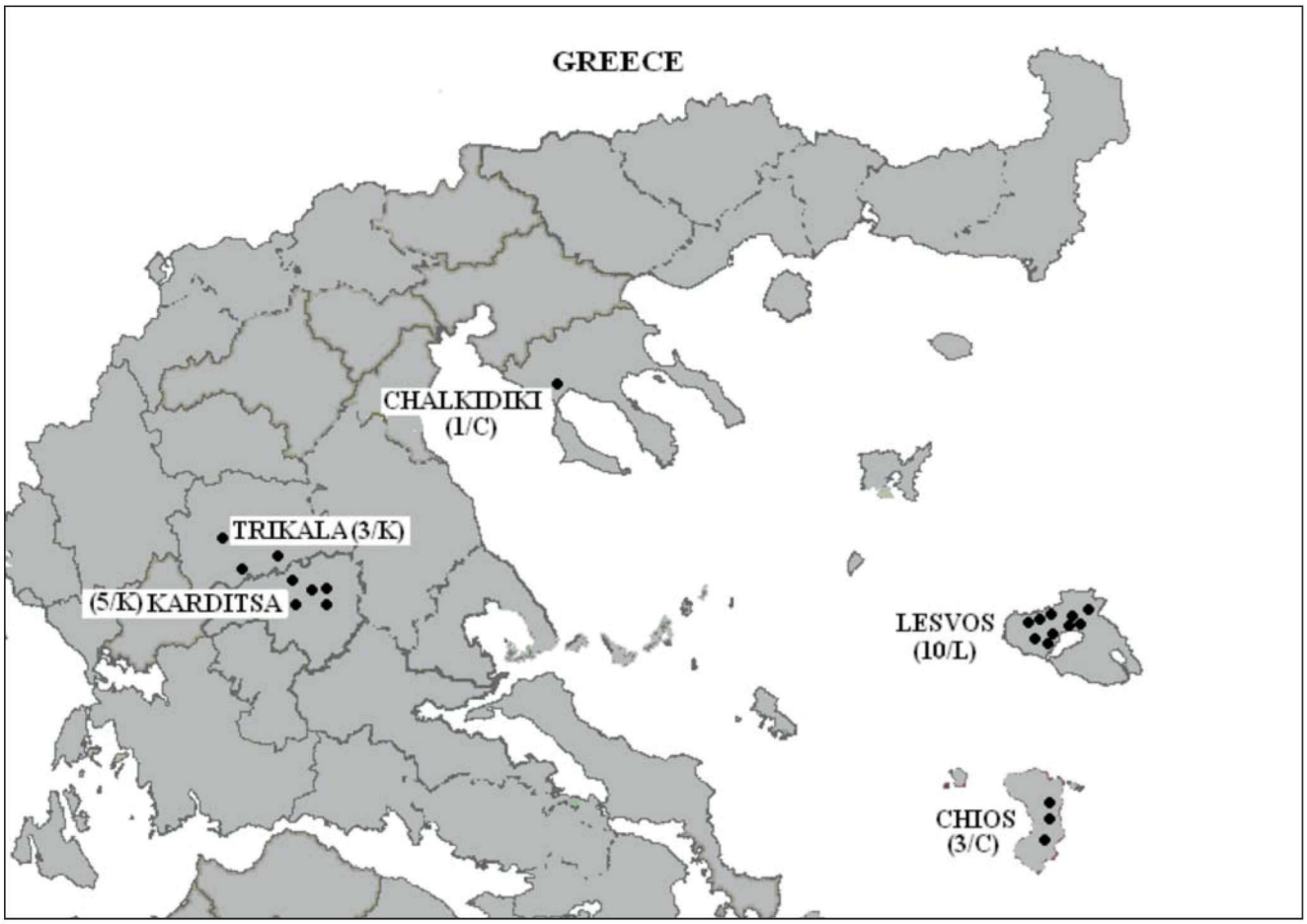
Characterization of animal genetic resources is a prerequisite for successful management programmes and informed decision making in national livestock development. The main objective of an advisable management of breeds should be to maintain both breed genetic diversity and adaptation to local conditions.

Thus, knowledge of the between-breeds genetic differentiation and of the within-breed genetic structure provides important insights into the genetic management of the breeds.

First, it allows the identification of pure-bred populations; the conservation of these populations is of high importance in order to maintain local adaptations. The recognition of the importance of these locally adapted genetic resources, as well as the need to improve characterization of the breeds' adaptive potential, is growing, in view of the increasing challenges of climate change (FAO, 2007).

Second, this knowledge is necessary so as to implement adequate mating schemes between genetically distant animals in order to maintain genetic diversity and decrease probable fertility and consanguinity problems related to increase inbreeding rates (Khaldi et al. 2010). Especially in geographically isolated regions, these inbreeding phenomena can lead the populations to baneful disappearance (Soulé and Mills, 1998).

This paper deals with the above questions for three dairy sheep breeds of Greece, namely the Lesvos, Karagouniko and Chios breeds (Map 1). The study of these breeds is of great interest, since they are raised in different regions and have a great economic importance as they are widely used in the country. According to the most recent data from the Hellenic Ministry of Rural Development and Food (Directorate of Inputs to Animal Production, 2009), the size of the pure-bred populations of these breeds is of 300.000, 200.000 and 56.000, for Lesvos, Karagouniko and Chios, respectively. Pedigree and performance recording are carried




**Map 1.** Origin of sheep samples

out in the three studied breeds in the frame of the Rural Development Programme 2007-2013.

The Chios breed originates from the island of Chios and it is spread in Northern Greece and, especially, in the regions of Macedonia and Thessaly. The breed is well-known for its high milk yield and prolificacy and it is extensively used for the upgrading of the local populations (Ligda et al. 2009). This semi fat-tailed breed with fine wool quality is, also, found in some areas of the western Turkish coast (under the name of Sakiz), in Cyprus, North Africa, the Middle East and other countries (Hatziminaoglou et al. 1996).

The Karagouniko breed originates from Central Greece and is mainly raised in the region of Thessaly. It is a typical lowland breed of thin-tailed mixed-wool type. These sheep are distinguished for their relatively high milk production and adaptation to marginal conditions (Katsaounis 1996, Zigogianis 1999, Georgoudis et al. 1995, Hatziminaoglou 2001, Rogdakis 2002).

The Lesvos breed is raised on the island of Lesvos. The breed is classified as fat-tailed and mixed-wool type of sheep. The breed is well-adapted in the dry climate and thermic conditions of the island, it is abstemious and exploits well the poor pasture of the mountains (Papavasiliou et al. 1998, Hatziminaoglou 2001, Rogdakis 2002). Due to its relatively high production, animals have been transferred and raised in other regions of Greece, as Peloponnesus, Sterea Ellada, Eastern Macedonia and Thraki and in the other islands of the Aegean Sea (Hatziminaoglou 2001).

The study of genetic diversity and structure of these breeds was conducted by means of the Random Amplified Polymorphic DNA-PCR method (RAPD-PCR). RAPD-PCR is a powerful molecular technique for the detection of genetic variability in different breeds or populations (Cushwa and Medrano 1994). It is a very convenient and easy-to-use method due to its speed in exporting results and the low cost of required reagents (Benter et al. 1995, Radu et al. 2001,

Chattopadhyay 2003), while it does not require the knowledge of nucleotide sequences of the DNA under consideration for the planning of required primers (Benieri 2005).

For these reasons, this technique has been extensively used for the investigation of genetic diversity (Rahman et al. 2006, Elmaci et al. 2007, Kumar et al. 2008, Budak and Çakır 2009, Khaldi et al. 2010), the identification of sex specific markers (Cushwa and Medrano 1994, Kumar et al. 2004, Kumar et al. 2008), the taxonomic identification (Abdel-Rahman and Hafez 2007), the estimation of genetic relationships (Paiva et al. 2005, Devrim et al. 2007, Hlophe S.R. 2011), and the assessment of inbreeding (Bhattacharya et al. 2003).

## MATERIALS AND METHODS

### Technique RAPD-PCR

The RAPD-PCR technique was used aiming at the determination of genetic structure and diversity of three breeds, as well as for the estimation of their genetic relation and distance. In addition, several studies, using this technique, revealed the close phylogenetic relationship of various breeds of sheep (Paiva et al. 2005, Kumar et al. 2008, Khaldi et al. 2010).

However, this technique is sensitive to minor changes or alterations in the reaction conditions (Budak and Çakır 2009); moreover, the use of Taq DNA polymerase from different companies affects the score of the produced fingerprints (Meunier and Grimont 1993). Thus, and in order to avoid changes or alterations due to the laboratorial handlings, we chose a Taq DNA polymerase of high specificity with less background (Platinum Taq Polymerase/Invitrogen Corporation) for better and more representative results.

### Animal Sampling and DNA Extraction

One hundred and twenty (120) blood samples of the three studied breeds were collected randomly (forty adult animals per breed) from four different geographical areas: a) from the island of Lesvos, samples of Lesvos breed, (L/10), b) from the island of Chios (C3) and from Chalkidiki (C1), samples of Chios breed, and c) from Karditsa (K5) and Trikala (K3), samples of Karagouniko breed (Map 1). All sampled animals had pedigree information from the Animal Genetic Improvement Center or from the National Agriculture Research Foundation.

With regard to the Lesvos breed, the samples were collected from ten different farms situated on the island: one farm from Pelopi (PL), three from Agia Paraskevi (AP a, b, c), three from Skaloxori (SK a, b, c), two from Parakoila (PR a, b) and one from Agra (AG), villages in the island of Lesvos. As for the Chios breed samples, they were collected from three farms situated on the island (IC a, b, c) and from one farm in Chalkidiki/Research Unit of the National Agriculture Research Foundation (FC). Finally, the samples of the Karagouniko breed were collected from three farms in Trikala: Megala Kalivia (MK), Palaiomonastiro (PM), Dentroxori (DX) and from five farms in Karditsa: Kalogriana (KG), Gourgouvitis (GV), Mirini (MR), Ag.Theodoros (AT) and the Research Unit of the National Agriculture Research Foundation (FK). Total DNA was isolated using the Genomic DNA Purification kit (Invitrogen Corporation).

### Primers and PCR amplification

Eleven (11) random primers were selected for this study and they were all used to estimate the genetic diversity and phylogenetic relationships among the three Greek sheep breeds. The nucleotide sequence of each primer was chosen within the constraints that the primer was 9 or 10 nucleotides in length, between 50% and 80% G+C in composition, and contained no palindromic sequences (Williams et al. 1990), except for two primers which were 40% G+C in composition (Table1). Primers 1, 2 and 17 from Ali, B.A. (2003) are referred as ALI-01, ALI-02 and ALI-17.

**Table 1.** List of RAPD - Primers with their nucleotide sequences and their contents in guanine and cytosine.

| No | Primer | Sequence ( 5' - 3') (forward, reverses) | G +C Content (%) |
|---|---|---|---|
| 1 | ALI -01 | ATG ACG TTG A | 40.0 |
| 2 | ALI -02 | GGG CTA GGG T | 70.0 |
| 3 | ALI -17 | GGT GAC GCA GGG GTA ACG CC | 70.0 |
| 4 | OPA -01 | CAG GCC CTT C | 70.0 |
| 5 | OPA -02 | TGC CGA GCT G | 70.0 |
| 6 | OPA-16 | AGC CAG CGA A | 60.0 |
| 7 | OPA -20 | GTT GCG ATC C | 60.0 |
| 8 | P -12 | CCC AGC GTT | 66.7 |
| 9 | P- 31 | CAC AGA GGG A | 60.0 |
| 10 | P -50 | TGC TAA TAC CGT ATG TGC TT | 40.0 |
| 11 | OPF- 05 | CCG AAT TCC C | 60.0 |

The Polymerase chain reaction (PCR) amplifications were performed in 15 μl reaction mixtures containing: 7.5μl Taq DNA polymerase-Platinum qPCR Super Mix-UDG (Invitrogen Corporation, Carlsbad, CA), 0.5-0.7 μl of Primer (10 pmol) and 1 μl of template DNA ( ~ 25 ng), per each PCR tube.

Three different protocols in Thermal Cycler were used:

a) Reaction mixtures containing ALI-01, ALI-02 and ALI-17 primers followed a protocol of initial denaturation at 95°C for 2 min, 45 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min (Ali 2003).

b) Reaction mixtures containing OPA-01, OPA-02, OPA-16, OPA-20, P12, P31 and P50 primers followed a protocol of initial denaturation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min (Devrim et al. 2007).

c) Reaction mixtures containing OPF-05 primer followed a protocol of initial denaturation at 94°C for 2 min, 40 cycles of denaturation at 49°C for 1 min, annealing at 35°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min (Budak and Çakır 2009).

Amplification products were separated by agarose gel (1.5%) electrophoresis at 100V. The 100 bp DNA Ladder (Invitrogen Corporation) was used as a molecular size marker. DNA fragments were then visualized by staining with ethidium bromide (1.5 μg $ml^{-1}$).

### Scoring of bands and statistical analysis

For the statistical analysis of the genetic data, the DNA bands were scored according to presence (1) and absence (0). The number of observed alleles (Na), effective number of alleles (Ne), Nei's genetic diversity (h) and Shannon's Information index (I) were estimated using the population genetic analysis software POPGENE Version 1.31 (Yeh et al. 1999). The phylogenetic relationships between breeds were analyzed by generating dendrograms between the three sheep breeds and between the farms, based on Nei's genetic distances (Nei, 1978) with unweighed pair group analysis using arithmetic average (method UPGMA) using POPGENE 1.31.

The Gst value was calculated using the format (Ht - Hs) / Ht, which can be used to explain the population genetic differentiation and the relationships among different strains (Nei 1987).

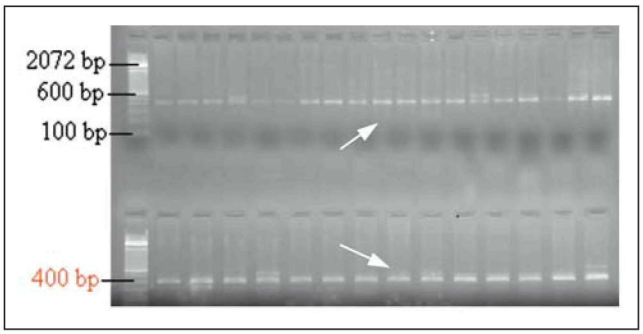



**Figure 1.** A band showing clearly per individual of Karagouniko sheep breed with ALI-01 primer in 400 bp length.

## RESULTS

### Polymorphisms

Eleven (11) oligonucleotide random primers were used to investigate genomic variability among the Lesvos (L), Chios (C) and Karagouniko (K) sheep breeds. All the primers, except for ALI-01 in Lesvos and Chios breeds, revealed polymorphic bands. This finding separates clearly the Karagouniko breed from Lesvos and Chios breeds. Figure 1 shows DNA fingerprints of ALI-01 primer. All the individuals (n=40) of Karagouniko breed presented a specific allele in 400 bp length, which is characteristic of the breed.

Moreover, the results from P31 primer showed a significant genetic separation of Karagouniko breed from Chios and Lesvos breeds: there was a specific allele (250 bp) in almost all samples of the two breeds (L=95% and C=97.5%) and it was absent from all samples of the Karagouniko breed (Fig. 2a.b.c).

The number of bands amplified from the 11 primers varied from 1 to 8 (Lesvos and Chios breeds) and from 1 to 7 (Karagouniko breed). The bands size ranged from 180 to 1100 bp (Lesvos), from 150 to 1400 bp (Chios) and from 180 to 1200 bp (Karagouniko) lengths. Most of the bands were identical between individuals of Lesvos and Chios breeds. This inference shows the close phylogenetic relationship among the two breeds.

In order to score fingerprints, we assumed one band corresponded to one locus. The % proportion of polymorphic loci for the 77 loci studied was 79.22%,

**Table 2.** List of RAPD primers used in determining genetic diversity within and among three populations of Greek sheep breeds. Lesvos (L), Chios (C), Karagouniko (K).

| Total number of scored bands | | | | | | Polymorphic bands (%) | | | | | | | Bands size range (bp) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Breed | | L | C | K | Total | L | C | K | Total | L | C | K | L | C | K |
| No | Primer | | | | | | | | | | | | | | |
| 1 | ALI-01 | 0 | 0 | 48 | 48 | 0 | 0 | 4 | 4 | 0,00 | 0,00 | 100,00 | 0 | 0 | 300-700 |
| 2 | ALI-02 | 113 | 113 | 113 | 339 | 3 | 3 | 2 | 8 | 100,00 | 100,00 | 66,67 | 300-450 | 300-450 | 300-450 |
| 3 | ALI-17 | 182 | 211 | 137 | 530 | 7 | 9 | 4 | 20 | 77,78 | 100,00 | 44,44 | 200-800 | 200-1400 | 200-400 |
| 4 | OPA-01 | 135 | 121 | 154 | 410 | 5 | 6 | 3 | 14 | 83,33 | 100,00 | 50,00 | 200-700 | 200-900 | 200-700 |
| 5 | OPA-02 | 177 | 140 | 177 | 494 | 8 | 9 | 4 | 21 | 88,89 | 100,00 | 44,44 | 250-1100 | 150-1100 | 250-1000 |
| 6 | OPA-16 | 111 | 50 | 59 | 220 | 6 | 5 | 3 | 14 | 85,71 | 71,43 | 42,86 | 200-800 | 200-1200 | 200-600 |
| 7 | OPA-20 | 145 | 142 | 229 | 516 | 5 | 5 | 1 | 11 | 55,56 | 55,56 | 11,11 | 300-900 | 300-900 | 200-800 |
| 8 | P-12 | 94 | 98 | 95 | 287 | 5 | 5 | 5 | 15 | 83,33 | 83,33 | 83,33 | 200-800 | 200-800 | 200-1200 |
| 9 | P-31 | 165 | 187 | 184 | 536 | 7 | 7 | 5 | 19 | 87,50 | 87,50 | 62,50 | 200-1000 | 200-1000 | 400-1200 |
| 10 | P-50 | 211 | 234 | 223 | 668 | 6 | 2 | 3 | 11 | 100,00 | 33,33 | 50,00 | 180-800 | 180-800 | 180-800 |
| 11 | ORF-05 | 215 | 144 | 199 | 558 | 9 | 6 | 5 | 20 | 90,00 | 60,00 | 50,00 | 200-1000 | 300-1000 | 300-1000 |
| 12 | Total | 1548 | 1440 | 1618 | 4606 | 61 | 57 | 39 | 157 | 79.22 | 74.03 | 55.65 | 180-1100 | 180-1400 | 180-1200 |
| Sample Size | | 40 | 40 | 40 | 120 | Loci: 231 | | | | Average: 67.96% | | | 150-1400 | | |

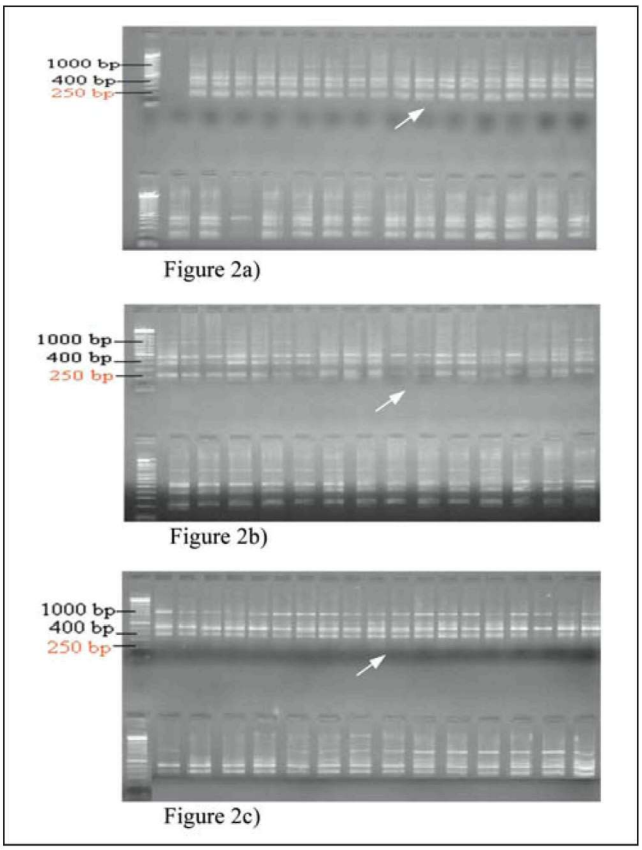



**Figure 2.** RAPD products profile were observed using P31 primer. Figures 2a & 2b of Lesvos and Chios sheep breeds, respectively, show bands in 250 bp length, whereas in the figure 2c of Karagouniko breed they are absent.

74.03% and 50.65% for the Lesvos, Chios and Karagouniko breeds, respectively (Table 2).

The highest number of polymorphic bands was 9 (OPF-05 in Lesvos and ALI-17, OPA-02 in Chios) and the lowest polymorphic band was 1 (OPA-20 in Karagouniko). The highest percentages of polymorphic bands were observed at ALI-02, OPA-02, P-50 and OPF-05 (89%-100%) in Lesvos breed, at ALI-02, ALI-17, OPA-01 and OPA-02 (100%) in Chios breed and at ALI-01 and P-12 (83%-100%) in Karagouniko breed. The lowest percentages of the polymorphic bands were found at OPA-20 primer in Lesvos (56%) and Karagouniko (11%) and at P-50 in Chios breed (33%). The different percentages of the polymorphic bands between primers and breeds confirmed the assumption that variation may exist between individuals of the same species (Appa Rao et al. 1996, Kumar et al. 2008).

The maximum number of bands was counted in Chios breed (5.85±0.36) and at OPF-05 primer (5.57± 0.80), while the minimum number of bands was recorded in Karagouniko breed (1.20±0.46) at OPA-16 primer (1.83±1.05). Table 3 shows the difference in the number of bands scored between the breeds and the primers. However, this variability was not sufficient to segregate Lesvos from Chios breed.

**Table 3.** Average number of bands (± Standard Deviation) among the three sheep breeds for the eleven primers. Maximum number and minimum number of bands per breed are presented.

| | | Average number of bands (± Standard Deviation) | | | |
|---|---|---|---|---|---|
| **No** | **Primers (L)** | **Lesvos (C)** | **Chios (K)** | **Karagouniko** | **Total Average** |
| 1 | ALI-01 | - | - | 1,20 ±0,46 | - |
| 2 | ALI-02 | 2,83±0,54 | 2,83±0,54 | 2,83±0,38 | 2,83±0,49 |
| 3 | ALI-17 | 4,55±1,38 | 5,28±1,70 | 3,43±0,59 | 4,42±1,22 |
| 4 | OPA-01 | 3,38±1,35 | 3,03±2,19 | 3,85±0,65 | 3,42±1,40 |
| 5 | OPA-02 | 4,43±2,27 | 3,50±2,22 | 4,43±0,80 | 4,12±1,77 |
| 6 | OPA-16 | 2,78±1,25 | 1,25±0,97 | 1,48±0,92 | 1,83±1,05 |
| 7 | OPA-20 | 3,63±1,24 | 3,55±1,47 | 5,73±0,45 | 4,30±1,05 |
| 8 | P-12 | 2,35±1,46 | 2,45±1,52 | 2,38±1,26 | 4,65±1,78 |
| 9 | P-31 | 4,13±1,52 | 4,68±1,23 | 4,60±0,80 | 2,39±1,41 |
| 10 | P-50 | 5,28±1,30 | 5,85±0,36 | 5,58±0,74 | 4,47±1,18 |
| 11 | OPF-05 | 5,38±1,73 | 3,60±2,36 | 4,98±1,23 | 5,57±0,80 |
| **Average:** | | **8** | **8** | **7** | **-** |
| **Min no. of bands:** | | **1** | **1** | **1** | **-** |

**Table 4.** Genetic diversity over all loci for the Lesvos, Chios and Karagouniko sheep breeds. Na = Observed number of alleles, Ne = Effective number of alleles (Kimura and Crow 1964), h = Nei's gene diversity, I = Shannon's Information index.

| Breed Name | Sample Size | Na ( n ± SD ) | Ne ( n ± SD ) | h ( n ± SD ) | I ( n ± SD ) |
|---|---|---|---|---|---|
| Lesvos | 40 | 1.79 ± 0.40 | 1.35 ± 0.33 | 0.21 ± 0.17 | 0.33 ± 0.24 |
| Chios | 40 | 1.74 ± 0.44 | 1.38 ± 0.38 | 0.22 ± 0.19 | 0.34 ± 0.26 |
| Karagouniko | 40 | 1.50 ± 0.50 | 1.20 ± 0.28 | 0.12 ± 0.16 | 0.20 ± 0.24 |

## Genetic Diversity and Distances

The observed and effective numbers of alleles were estimated over all loci. The number of observed alleles (Na) ranged from 1.50 (Karagouniko) to 1.79 (Lesvos), while the effective number of alleles (Ne) ranged from 1.20 (Karagouniko) to 1.38 (Chios) and it was lower than Na, as expected (Budak and Çakır 2009). The highest values of Nei's genetic diversity (h) and Shannon's Information index (I) - 0.22 and 0.34, respectively - were observed in Chios breed, while the lowest values - 0.12 and 0.20, respectively - were observed in the Karagouniko breed (Table 4).

All genetic data of 11 RAPD primers were pooled for the estimation of genetic distance (GD) and genetic identity (GI). The highest genetic distance (GD=0.1979) was observed between Chios and Karagouniko and the lowest (GD=0.0376) between Chios and Lesvos. Similarly, the highest genetic identity (GI=0.9631) was found between Chios and Lesvos and the lowest (GI=0.8204) between Chios and Karagouniko. The above results indicate the close relationship between Chios and Lesvos breeds. Nei's genetic distance and genetic identity of breeds are given in Table 5.

**Table 5.** Unbiased Measure (Nei 1978) of Genetic distance (below diagonal) and genetic identity (above diagonal) based on RAPD statistical analysis among three Greek sheep breeds.

| Breeds | Chios | Karagouniko | Lesvos |
|---|---|---|---|
| Chios | **** | 0.8204 | 0.9631 |
| Karagouniko | 0.1979 | **** | 0.8444 |
| Lesvos | 0.0376 | 0.1691 | **** |

The dendrogram in figure 3 shows that the three

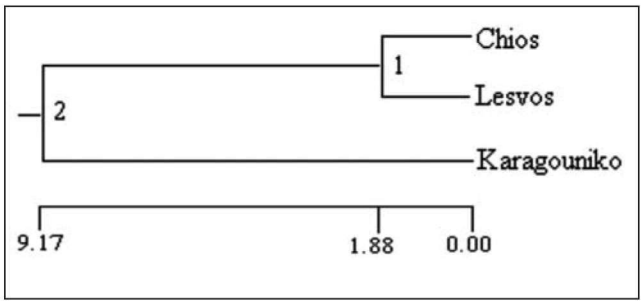



**Figure 3.** Dendrogram among the three Greek sheep breeds, based on Nei's (1978) genetic distance and constructed using UPGMA analysis through neighbour procedure of PHYLIP Version 3.5 (POPGEN software).

breeds can be separated into two distinct groups. One group is constituted by Chios and Lesvos breeds and the other by the Karagouniko breed.

### Genetic Structure

The RAPD data were used to construct a dendrogram among the sampled farms. The genetic relationships among the twenty two (22) flocks using the information gathered from 231 RAPD loci were calculated. The genetic similarity among all flocks with their abbreviations is given in figure 4.

Although Chios and Lesvos breeds are closely related, their flocks are clearly clustered in separated groups of flocks of the same breed. This denotes that the breeds are genetically distinct, despite their low genetic distance. As for the Karagouniko breed, the dendrogram shows that it is completely distant from the other two breeds and it is separated in one cluster.

One interesting finding emerges for the dendrogram: The Lesvos flocks can be separated into two groups: group (a) is comprised of PL, Ska, Skb, PRb, APc flocks and group (b) is comprised of APa, APb, SKc, PRa and AG flocks. Geographic distance between the flocks cannot be used as an explanatory factor for the separation of the two groups, as flocks from the same village appear to belong to separate groups (SK and PR flocks). Group (a) appears to be more closely related to the Chios flocks than group (b). This can be due to some cross-breedings that occurred

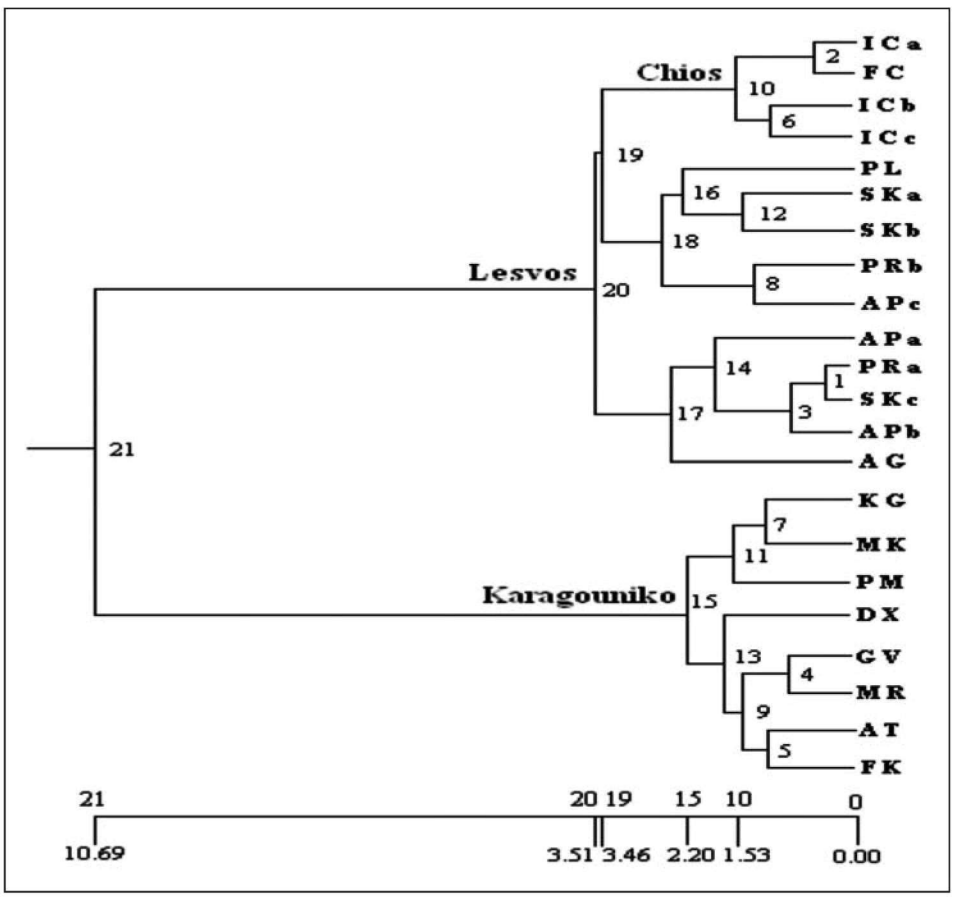



**Figure 4.** Dendrogram among twenty two (22) flocks of the three Greek sheep breeds based on Nei's (1978) genetic distance (POPGEN software -Version 1.31). a) Lesvos breed samples L1-L10: Pelopi (PL), Agia Paraskevi (AP a, b, c), Skaloxori (SK a, b, c), Parakoila (PR a, b) and Agra (AG), all villages in the island of Lesvos. b) Chios breed C1 and C3: Research Unit of Chalkidiki/ National Agriculture Research Foundation (FC) and Chios island (IC a, b, c). c) Karagouniko breed K3 and K5: Megala Kalivia (M K), Palaiomonastiro (PM), Dentroxori (DX), Kalogriana (KG), Gourgouvitis (GV), Mirini (MR), Ag.Theodoros (AT) and from the Research Unit of National Agriculture Research Foundation of Karditsa (FK).

**Supplementary Table 1.** Unbiased Measures (Nei 1978) of Genetic distances (below diagonal) and genetic identities (above diagonal). The lowest values GDs and highest GIs among of flocks are printed in bold.

| Br. | | Chios | | | | Karagouniko | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Herbs | I Ca | I Cb | I Cc | F C | K G | M K | P M | D X | G V | M R | A T | F K |
| Chios | I Ca | **** | 0.9671 | 0.9715 | **0.9899** | 0.8222 | 0.8204 | 0.7987 | 0.8157 | 0.8012 | 0.7957 | 0.8099 | 0.8221 |
| | I Cb | 0.0335 | **** | 0.9767 | 0.9724 | 0.8003 | 0.8004 | 0.7986 | 0.7934 | 0.7870 | 0.7761 | 0.7672 | 0.7812 |
| | I Cc | 0.0289 | 0.0236 | **** | 0.9682 | 0.8185 | 0.7982 | 0.8041 | 0.8014 | 0.7962 | 0.7859 | 0.7798 | 0.8001 |
| | F C | **0.0102** | 0.0280 | 0.0323 | **** | 0.8097 | 0.7996 | 0.7836 | 0.8225 | 0.8101 | 0.7964 | 0.7997 | 0.8182 |
| Karagouniko | K G | 0.1958 | 0.2228 | 0.2003 | 0.2111 | **** | 0.9744 | 0.9679 | 0.9536 | 0.9649 | 0.9640 | 0.9614 | 0.9697 |
| | M K | 0.1979 | 0.2227 | 0.2254 | 0.2236 | 0.0259 | **** | 0.9698 | 0.9477 | 0.9619 | 0.9546 | 0.9568 | 0.9612 |
| | P M | 0.2248 | 0.2249 | 0.2180 | 0.2439 | 0.0326 | 0.0306 | **** | 0.9473 | 0.9445 | 0.9608 | 0.9577 | 0.9471 |
| | D X | 0.2037 | 0.2315 | 0.2213 | 0.1954 | 0.0475 | 0.0537 | 0.0541 | **** | 0.9677 | 0.9705 | 0.9684 | 0.9570 |
| | G V | 0.2217 | 0.2396 | 0.2279 | 0.2106 | 0.0357 | 0.0388 | 0.0570 | 0.0329 | **** | **0.9826** | 0.9621 | 0.9695 |
| | M R | 0.2285 | 0.2535 | 0.2409 | 0.2276 | 0.0367 | 0.0464 | 0.0400 | 0.0300 | **0.0176** | **** | 0.9815 | 0.9672 |
| | A T | 0.2109 | 0.2650 | 0.2487 | 0.2235 | 0.0394 | 0.0442 | 0.0432 | 0.0321 | 0.0387 | 0.0186 | **** | 0.9770 |
| | F K | 0.1959 | 0.2469 | 0.2231 | 0.2007 | 0.0308 | 0.0396 | 0.0543 | 0.0440 | 0.0310 | 0.0333 | 0.0233 | **** |
| Lesvos | P L | 0.0586 | 0.0743 | 0.0963 | 0.0588 | 0.1676 | 0.1753 | 0.1707 | 0.1727 | 0.1614 | 0.1771 | 0.1733 | 0.1625 |
| | S K a | 0.0504 | 0.0481 | 0.0707 | 0.0447 | 0.1996 | 0.1960 | 0.2085 | 0.1816 | 0.2083 | 0.2223 | 0.2230 | 0.2248 |
| | A P a | 0.0491 | 0.0463 | 0.0464 | 0.0521 | 0.1786 | 0.2081 | 0.2097 | 0.1982 | 0.1865 | 0.2103 | 0.2132 | 0.1884 |
| | P R a | 0.0738 | 0.0504 | 0.0725 | 0.0790 | 0.2166 | 0.2043 | 0.1756 | 0.2271 | 0.2324 | 0.2429 | 0.2483 | 0.2396 |
| | A G | 0.1442 | 0.1101 | 0.1138 | 0.1367 | 0.2130 | 0.2403 | 0.2203 | 0.2681 | 0.2226 | 0.2745 | **0.2992** | 0.2580 |
| | S K b | 0.0638 | 0.0840 | 0.0894 | 0.0584 | 0.1965 | 0.1871 | 0.2073 | 0.2022 | 0.1959 | 0.2156 | 0.2220 | 0.1953 |
| | A P b | 0.0655 | 0.0465 | 0.0488 | 0.0717 | 0.2049 | 0.2090 | 0.1903 | 0.2242 | 0.2190 | 0.2257 | 0.2408 | 0.2337 |
| | S K c | 0.0573 | 0.0493 | 0.0560 | 0.0645 | 0.2131 | 0.2229 | 0.2038 | 0.2318 | 0.2311 | 0.2508 | 0.2594 | 0.2400 |
| | P R b | 0.0713 | 0.0797 | 0.0853 | 0.0559 | 0.1933 | 0.2171 | 0.2221 | 0.1915 | 0.1861 | 0.2030 | 0.2109 | 0.1713 |
| | A P c | 0.0857 | 0.0714 | 0.0688 | 0.0686 | 0.1828 | 0.1986 | 0.1901 | 0.1937 | 0.1832 | 0.2255 | 0.2459 | 0.1981 |

in the past, according to old references, among Lesvos ewes and Chios rams in the island of Lesvos, which today appear as genetic remnant.

However, despite the proximity of Lesvos and Chios breeds, it is assumed that the same size fragments might be different in the nucleotide sequence or when comparing different breeds that show greater diversity, as the fragments of same size might have derived from non-allelic regions (Kumar et al. 2008).

The results of the dendrogram, also, revealed that while the genetic similarity among the flocks in Lesvos was irrelavant to the geographical distances between the sampling villages, these were relevant in Karagouniko breed. It is an expected result as farmers in the two regions follow different practices in choosing breeding rams, in Thessaly the farmers usually prefer rams from the same or neighbouring flocks. The genetic similarity between the ICa unit from the island of Chios and FC of the National Agriculture Research Foundation of Chalkidiki is easily explicable, since the latter was founded by individuals issued from Chios.

The genetic distances (GDs) and genetic identities (GIs) between the populations of flocks were analytically included in supplementary tables 1 and 2. The lowest GD (0.0065) was obtained between PRa and SKc flocks of Lesvos breed, whereas the highest (0.2992) was observed between AG flocks of Lesvos breed and AT flocks of Karagouniko breed. The lowest GDs were 0.0065, 0.0102 and 0.0176, while, on the contrary, the highest GIs were found at 0.9935, 0.9899 and 0.9826 for Lesvos, Chios and Karagouniko breeds, respectively.

The total variation (Ht) among all flocks was 0.2163 ± 0.0317, 0.1295 ± 0.0273 and 0.2138 ± 0.0410 for the Lesvos, Karagouniko and Chios breed, respectively, while the average variation within flocks (Hs) was

**Supplementary Table 2.** Unbiased Measures (Nei 1978) of Genetic distances (below diagonal) and genetic identities (above diagonal). The lowest values GDs and highest GIs among of flocks are printed in bold.

| Br. | Lesvos | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Herbs | P L | S K a | A P a | P R a | A G | S K b | A P b | S K c | P R b | A P c |
| Chios | I Ca | 0.9431 | 0.9508 | 0.9521 | 0.9289 | 0.8657 | 0.9382 | 0.9366 | 0.9443 | 0.9312 | 0.9179 |
| | I Cb | 0.9284 | 0.9530 | 0.9547 | 0.9509 | 0.8958 | 0.9194 | 0.9545 | 0.9519 | 0.9234 | 0.9311 |
| | I Cc | 0.9082 | 0.9317 | 0.9546 | 0.9300 | 0.8924 | 0.9145 | 0.9524 | 0.9455 | 0.9182 | 0.9335 |
| | F C | 0.9429 | 0.9563 | 0.9492 | 0.9240 | 0.8723 | 0.9432 | 0.9308 | 0.9376 | 0.9456 | 0.9337 |
| Karagouniko | K G | 0.8457 | 0.8191 | 0.8365 | 0.8053 | 0.8082 | 0.8216 | 0.8147 | 0.8081 | 0.8242 | 0.8329 |
| | M K | 0.8392 | 0.8220 | 0.8121 | 0.8152 | 0.7864 | 0.8294 | 0.8114 | 0.8002 | 0.8049 | 0.8199 |
| | P M | 0.8431 | 0.8118 | 0.8109 | 0.8389 | 0.8023 | 0.8127 | 0.8267 | 0.8156 | 0.8008 | 0.8268 |
| | D X | 0.8414 | 0.8339 | 0.8202 | 0.7969 | 0.7648 | 0.8169 | 0.7992 | 0.7931 | 0.8257 | 0.8239 |
| | G V | 0.8510 | 0.8119 | 0.8298 | 0.7926 | 0.8004 | 0.8221 | 0.8033 | 0.7937 | 0.8302 | 0.8326 |
| | M R | 0.8377 | 0.8007 | 0.8104 | 0.7844 | 0.7600 | 0.8061 | 0.7980 | 0.7782 | 0.8163 | 0.7981 |
| | A T | 0.8409 | 0.8001 | 0.8080 | 0.7801 | **0.7414** | 0.8009 | 0.7860 | 0.7715 | 0.8099 | 0.7820 |
| | F K | 0.8500 | 0.7986 | 0.8283 | 0.7869 | 0.7726 | 0.8226 | 0.7916 | 0.7866 | 0.8425 | 0.8203 |
| Lesvos | P L | **** | 0.9526 | 0.9578 | 0.9450 | 0.9023 | 0.9542 | 0.9386 | 0.9449 | 0.9453 | 0.9439 |
| | S K a | 0.0485 | **** | 0.9143 | 0.9450 | 0.8827 | 0.9687 | 0.9377 | 0.9316 | 0.9349 | 0.9487 |
| | A P a | 0.0431 | 0.0896 | **** | 0.9512 | 0.9583 | 0.9100 | 0.9659 | 0.9695 | 0.9391 | 0.9314 |
| | P R a | 0.0565 | 0.0566 | 0.0500 | **** | 0.9404 | 0.9516 | 0.9883 | **0.9935** | 0.9199 | 0.9472 |
| | A G | 0.1028 | 0.1247 | 0.0425 | 0.0615 | **** | 0.9120 | 0.9492 | 0.9582 | 0.9082 | 0.9408 |
| | S K b | 0.0469 | 0.0318 | 0.0944 | 0.0496 | 0.0921 | **** | 0.9461 | 0.9500 | 0.9571 | 0.9553 |
| | A P b | 0.0633 | 0.0643 | 0.0346 | 0.0117 | 0.0521 | 0.0554 | **** | 0.9798 | 0.9473 | 0.9462 |
| | S K c | 0.0566 | 0.0708 | 0.0309 | **0.0065** | 0.0427 | 0.0513 | 0.0204 | **** | 0.9337 | 0.9479 |
| | P R b | 0.0562 | 0.0673 | 0.0628 | 0.0835 | 0.0963 | 0.0438 | 0.0541 | 0.0686 | **** | 0.9731 |
| | A P c | 0.0578 | 0.0526 | 0.0710 | 0.0543 | 0.0610 | 0.0458 | 0.0553 | 0.0536 | 0.0273 | **** |

0.1549 ± 0.0161, 0.0912 ± 0.0145 and 0.1847 ± 0.0313 for L, K and C, respectively.

The mean gene differentiation (Gst) among flocks was estimated to 0.2840 (Lesvos), 0.2961 (Karagouniko) and 0.1361 (Chios).

## DISCUSSION

The RAPD-PCR method was used to study the relationship between three of the most widespread breeds of sheep of Greece and to conduct a preliminary assessment of the genetic diversity and structure of these breeds. Our analysis comprised two levels of the breeds' genetic structure: i) the genetic differentiation among the three breeds and ii) the genetic differentiation among the flocks within each breed. This combined approach gave two main findings.

First, the study of genetic distances revealed that the Karagouniko sheep breed is genetically distinct from Chios (GD=0.1979) and Lesvos (GD=0.1691), while the breeds of Chios and Lesvos are genetically similar (GI=0.9631). Furthermore, the study of polymorphic loci led us to an important finding, that of the particular indicators located in Karagouniko breed, as definitional datum of genetic identity or as a fingerprint of breed. The present study suggests, therefore, that the RAPD-DNA methods can be an efficient tool for the determination of phylogenetic relationships and genetic identity among sheep breeds.

Second, the genetic structure of the flocks revealed that, although the flocks of Chios and Lesvos breeds are clustered in separated groups, half of the flocks (n=5) of Lesvos have a relatively closer relationship with those of Chios than with the other Lesvos flocks. It must be underlined that this is the first study that reports the close genetic relationship between the Chios and Lesvos breeds and gives strong evidence to

hypotheses about their related origin.

The close relationship between Lesvos and Chios found in the present study is confirmed by older reports, where Lesvos sheep breed originates from cross-breeds among thin-tailed local sheep of the island of Lesvos with either the sheep of Chios breed from the island of Chios or the fat-tailed sheep of the Turkish Kamakuyruk breed from Anatolia (Hatziloos 1941, Dimitriadis 1957, Karantounias 1964, Katsaounis 1996, Papavasiliou et al. 1998, Zygogianis 1999, Hatziminaoglou 2001, Ministry of Rural Development and Food 2004). Moreover, the genetic distance of Lesvos and Chios breeds has been confirmed by Handley et al. (2007) with the use of microsatellites. Although the technique differs from the RAPD-PCR technique used in the present work, both result that Lesvos and Chios breeds are genetically distinguished.

The flocks sampled in our study showed significant genetic differentiation for both Karagouniko and Lesvos breeds. However, the pattern of differentiation was very different according to the breed: while more distant flocks showed higher genetic distance for the Karagouniko breed, the genetic differentiation of Lesvos flocks did not show any clear pattern with geographic distance; geographically adjacent flocks seem to belong to separate groups [see dendrogram group (a) and (b) Fig.4]. Further investigation is needed in order to disentangle the reasons behind the observed pattern of genetic differentiation in the island of Lesvos (e.g. mating schemes promoted by local farmers).

In contrast, the flocks of Chios breed showed lower genetic differentiation. This is likely due to our sampling protocol: i) the low number of flocks sampled and ii) the fact that the FC flock was founded by the island flocks. In a future work, more samples will be collected from the region of Central Macedonia, where the majority of Chios sheep population is raised.

The measure of genetic diversity within flocks (mean heterozygosity, Hs) showed that the Chios flocks are more genetically diverse than the flocks of the other two breeds. A further analysis with samples collected from the region of Central Macedonia could give detailed explanations.

Genetic variation within and among breeds, revealed by molecular techniques, provide useful data, which, in combination with pedigree information, could have important implications for the genetic improvement programmes of the Greek sheep breeds and for the conservation of these breeds. In order to have a more complete view on the management and conservation of these economically important breeds, further study is needed to link genetic and phenotypic variation with environmental parameters. This is necessary to assess the degree of local adaptations and propose adequate breeding strategies.

## CONCLUSIONS

The combined approach of our methodology gave us results that the Karagouniko breed is genetically distinct from Chios (GD=0.1979) and Lesvos (GD= 0.1691), while Chios and Lesvos breeds are genetically similar (GI=0.9631). Although Chios and Lesvos breeds have origin from neighbour islands, it is the first study that reports the close genetic relationship between the two breeds and gives strong evidence to hypotheses about their related origin. Furthermore, the study of polymorphic loci revealed particular indicators located in Karagouniko breed, as definitional datum of genetic identity or as a fingerprint of breed.

In conclusion, genetic variation and relationship within and among breeds, revealed by RAPD-PCR, provide useful data, which, in combination with pedigree information, could have important implications for the genetic improvement programmes of the Greek sheep breeds and for the conservation of these breeds. RAPD-DNA method can be an efficient tool for the determination of phylogenetic relationships and genetic identity among sheep breeds.

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