Association of subclinical mastitis prevalence with sheep breeds in Greece

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Objective was to describe potential associations of subclinical mastitis with sheep breeds in Greece. A countrywide survey (2,198 ewes in 111 farms) was performed. Prevalence of subclinical mastitis was 0.260. Results did not indicate difference in prevalence of subclinical mastitis between farms with pure-bred and farms with cross-bred animals, nor difference in prevalence between farms with Greek pure-bred animals and farms with imported pure-bred animals. Results indicated that prevalence of subclinical mastitis was smaller in farms with Assaf-breed (0.100) and higher in farms with Frisarta-breed (0.625) (P<0.02). Prevalence of mastitis was smaller in farms with Greek traditional indigenous breeds (0.221) (P=0.007). In a model that included sheep breed and management system in farm, breed emerged of significance for prevalence of subclinical mastitis (P=0.003).

Key words: breed, management system, prevalence, subclinical mastitis, sheep

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Predominant type of sheep production in Greece is dairy, with various breeds present around the country. There has been no systematic study of potential association between sheep breeds in the country and mastitis. The genetic background of sheep susceptibility to mastitis has been presented and the role of sheep breeds, carriers of relevant genes, has been mentioned (Bishop, 2015). There is little work worldwide in relation to potential susceptibility of sheep breeds to mastitis: Larsgard and Vaabenoe (1993) have indicated some differences between Norwegian sheep breeds, whilst Burriel (1997) has reported that mule ewes were more susceptible to mastitis than Welsh-Mountain ewes (a traditional indigenous British breed). Objective of the work was to describe potential associations of subclinical mastitis with breeds of sheep in Greece.

Materials and methods

In total, 111 sheep farms in the 13 administrative regions of Greece were included into the study and visited for collection of samples and information. The investigators visited all farms for

sample collection. In each farm, 20 clinically healthy ewes were selected and sampled by use of standardised methods. Bacteriological and cytological examinations were performed in milk samples. Ewes were considered to have subclinical mastitis when a bacteriologically positive milk sample ([a] >10 colonies of the same organism and [b] no more than two different types of colonies) with concurrently increased CMT score (≥'1') plus neutrophil and lymphocyte proportion (≥65% of all leucocytes) was detected.

Mixed-effects logistic regression was employed, using the different farms as 'random effect'. Analysis of variance was employed for performing comparisons between farms in relation to prevalence of subclinical mastitis. Farms with Cephalonia, Crete, Karagouniko, Karystos, Lesvos and Vlahiko breeds were clustered as 'Greek traditional indigenous breeds' and comparisons were repeated. A multivariable model was created using mixed-effects logistic regression with farm as the random effect, which included as variables the management system and the sheep breed.

Detailed description of procedures and techniques employed are in Suppl. material 1. Location of farms around the country is shown in Suppl. material 2.

Results

In total, 2,220 ewes were examined and 2,198 were sampled. Among these, 572 were detected with subclinical mastitis; prevalence was 0.260 (95% C.I.: 0.242-0.279). Prevalence within farm varied from 0.000 to 0.850 (median: 0.250). The most frequently isolated bacteria from ewes with subclinical mastitis were *Staphylococcus* spp. (n = 531) (*Staphylococcus aureus* or coagulase-negative species). Less frequently isolated organisms were *Streptococcus* spp., *Corynebacterium* spp., *Escherichia coli*, *Micrococcus* spp., *Mannheimia haemolytica* and *Trueperella pyogenes*.

Of the 111 farms, 58 included pure-bred animals (33 with Greek breeds: Cephalonia n=2, Chios n=13, Crete n=4, Frisarta n=2, Karagouniko n=3, Karystos n=1, Lesvos n=5, Vlahiko n=3, and 25 with imported breeds: Assaf n=2, Lacaune n=23). The other 53 farms included cross-bred animals. In farms with intensive management system, pure-bred animals prevailed (17/26), of which most were imported (11/17). Pure-breeds also prevailed in semi-extensive or extensive management system (16/28), but most were Greek breeds (14/16). In farms with semi-intensive management system, cross-breeds prevailed (32/57). Details of breeds in farms are in Suppl. material 3.

Difference in prevalence of subclinical mastitis between farms with pure-bred and farms with cross-bred animals (0.276 and 0.243, respectively) was not significant (P=0.144). Difference in prevalence of subclinical mastitis between farms with Greek pure-bred animals and farms with imported pure-bred animals (0.284 and 0.265, respectively) was also not significant (P=0.240). Not significant was also the difference in prevalence between farms with imported pure-bred animals and all other farms (0.265 and 0.259, respectively) (P=0.125). Similarly, differences in prevalence between farms with Greek pure-bred animals, farms with imported pure-bred animals

and farms with cross-bred animals (0.284, 0.265 and 0.243, respectively) were not significant (P=0.123).

When farms with the various pure-breeds were considered, it became evident that prevalence of subclinical mastitis was significantly smaller in farms with Assaf-breed sheep and significantly higher in farms with Frisarta-breed sheep (P<0.02 for both comparisons). Further, there was significantly smaller prevalence in farms with Karystos-breed sheep (P=0.045) and a tendency for higher prevalence in farms with Chios-breed sheep (P=0.125). When farms with the six Greek traditional indigenous breeds were clustered together, it emerged that prevalence of subclinical mastitis was significantly smaller in that cluster (P=0.007). All other evaluations did not yield significant differences (P>0.250). Details are in Table 1 and Figure 1.

Sheep breed emerged from the multivariable mixed-effects model as the significant factor for the prevalence of subclinical mastitis (P=0.003). There was a trend for contribution by the management system (P=0.087); interactions between breed and management system were not important (P=0.845). Results were similar when calculations were performed, after including farms under semi-extensive and extensive management in one cluster (P=0.007, P=0.060, P=0.768, respectively).

Discussion

Lacaune- and Chios-breed animals are popular in the country. These are sheep with high milk production, thus of importance in the dairy production systems applied in the country, which explains the higher proportion of farms with these breeds. The findings indicate increased penetration of imported breeds, which, in recent years, have been favoured by Greek farmers. Lacaune and Assaf predominate among imported sheep breeds in Greece, as they are animals of increased milk production, higher than indigenous Greek breeds. These animals cannot be adapted to the environment, which is reflected in them being included in farms managed intensively or semi-intensively, where they are sheltered and their needs, especially nutritional requirements, can be controlled and covered. Nevertheless, uncontrolled imports may increase risk for transmission of diseases to the indigenous sheep population; for example, in Spain a large proportion of Assaf animals have been found to be infected with *Small Ruminant Lentivirus* (Minguijon et al., 2015), which may lead to transmission of the pathogen to uninfected flocks in Greece after import. Traditional breeds have also been identified, these being of limited geographical distribution and, mainly, in flocks managed under the semi-extensive or extensive system, which constitute the traditional shepherding forms in the country. These are low-input breeds, with very good adaptability to environmental conditions and able to make excellent use of natural resources and locally produced feedstuffs, which explains their increased frequency in farms managed semi-extensively or extensively (Georgoudis et al., 2011). There is evidence regarding genetic relationship between animals of those breeds (Ligda et al., 2009; Georgoudis et al., 2011), thus lending support to clustering these breeds for the statistical analysis.

Bacteriological examination of milk samples is employed for diagnosis of subclinical mastitis, as it is considered to provide precise and exhaustive information on infected mammary glands and pathogen involved. However, it is difficult to implement at a large scale and also has various limitations. Moreover, bacterial shedding is variable and levels may sometimes be too low to be detected by conventional techniques (Rupp and Foucras, 2010). Simple, indirect methods have also been widely applied, based on evaluation of inflammation. The ones most frequently used are somatic cell counting and various indirect tests for their measurement. A difficulty in using somatic cell counting is that factors known to influence somatic cell counting have different magnitude in healthy and infected animals (Detilleux and Leroy, 2000). Further, there is difference in types of cells in mammary secretion, which can provide an indication regarding the inflammation. Indeed, the associations between bacteria and somatic cells, particularly of the various types of leucocytes, can be used to better define the disease (Albenzio et al., 2009). The definition of subclinical mastitis used in this study (i.e., combination of a bacteriologically positive milk sample with increased CMT score plus high proportion of neutrophil and lymphocyte) takes that into account and was adopted to overcome shortcomings of the methods described previously.

Present results have indicated increased prevalence of subclinical mastitis in Friesarta-breed farms. Animals of the breed are high-yielding, but, in general, considered to be particularly susceptible to diseases, e.g., respiratory infections. Increased susceptibility to mastitis can be attributed to breed-specific impaired local defence mechanisms in the udder (Fragkou et al., 2007; 2010). Present findings provide field corroboration to the experimental evidence. Traditional Greek sheep breeds have shown reduced frequency of subclinical mastitis. In a broader sense, resistance could be defined as the ability to avoid any infection and/or the quick recovery from an infection (Rupp and Boichard, 2003) and involves different components: avoiding entry of the pathogen into the teat, mounting an immune response capable of limiting its development in the mammary gland and clearing the infection, as well as controlling the pathogenic effects of the infection, such as tissue damage (Rupp and Foucras, 2010). In Karagouniko ewes, lymphoid follicles have been identified in the teat duct and have been repeatedly shown to play a clear protective role against invading pathogens (Fragkou et al., 2010). Higher allocation of resources to defence mechanisms of ewes afforded by low milk production of these animals can also play a predominant role and contribute to efficient counteraction against invading mammary pathogens. A tendency of increased prevalence of subclinical mastitis in Chios-breed sheep has also emerged. Possible reasons could be the bad udder conformation, which hinders correct milking and contributes to infections (Gelasakis et al., 2012), and the innate peri-parturient immunosuppression associated with macrophage and neutrophil function (Theodorou et al., 2007). Previous studies on other breeds (e.g., Latxa and Sarda) have indeed shown favourable correlations between SCC and udder conformation (Legarra and Ugarte, 2005; Sechi et al., 2007), suggesting that udders with what is perceived to be a good shape would be less affected by subclinical mastitis. In addition, udders with bad conformation can predispose to development of mastitis (Gelasakis et al., 2012). Further, differences in somatic cell counts in milk of healthy animals recorded between sheep breeds (Rupp

and Foucras, 2010) can reflect the immunological competence of the respective mammary glands against invading microorganisms and the final result (Albenzio et al., 2012).

In cows, there are many studies detailing genetic resistance to mastitis (discussed by Fragkou et al., 2007). Differences to various defence determinants of susceptible/resistant animals have been reported, e.g. number of blood polymorphonuclear cells after calving, lactoferrin concentration, production of immunoglobulins, production of complement fragment C5a, production and mobilization of cytokines. There is also information regarding genetic control of lymphocyte mobilization and role, e.g. heritability (h^2) of T-cell proliferation ranges between h^2 = 0 to 0.40, genetic mechanisms have been identified for production of T-cell and B-cell receptor phenotypes.

Mastitis is a prime target disease to develop breeding for resistance and produce mastitis-resistant sheep (Davies, 2009; Bishop, 2015), as in sheep genomic selection has been shown to have good accuracy for mastitis resistance (Duchemin et al., 2012). The findings have provided evidence of associations of subclinical mastitis with breed, which have only rarely been reported. In Greece, the only breeding program for genetic control of diseases has been that for scrapie. Certainly, it is more difficult to select for resistance to mastitis, which is a polygenic trait, therefore, selection for a complex of traits is necessary, where many genes with small effects are involved. Given the significance of the sheep industry in the country and the importance of mastitis as a limiting factor in milk production, there is a need to consider genetic improvement for reduced susceptibility to mastitis, as a sustainable means to control of the disease.

Acknowledgments

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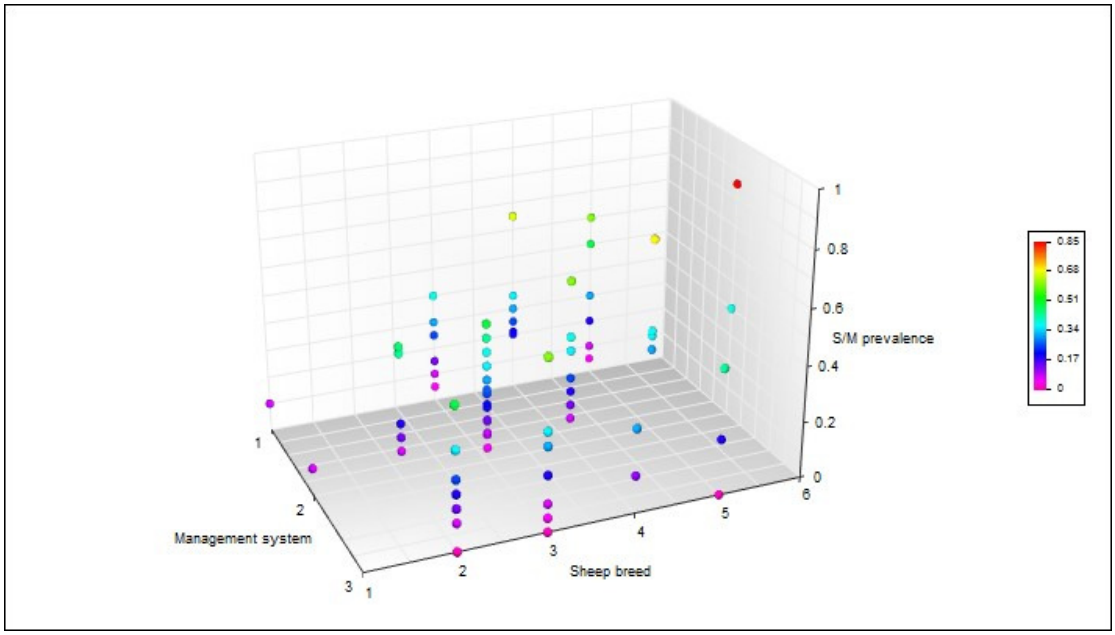
Figure 1. Scatter plot of results of subclinical mastitis prevalence (z axis) against management system applied in farms (x axis) and sheep breed (y axis) in 111 sheep farms in Greece.



Management system: 1: intensive, 2: semi-intensive, 3: semi-extensive or extensive.
Sheep breed: 1: Assaf, 2: Greek traditional indigenous breeds, 3: Cross-breeds, 4: Lacaune, 5: Chios, 6: Frisarta.
Subclinical mastitis prevalence: Colour map indicates prevalence of subclinical mastitis.

Table 1. Importance of breed in prevalence of subclinical mastitis in sheep in Greece.

(a) Greek breeds considered individually

Sheep breeds (no. of farms)	Prevalence	Odds ratio (95% CI)	*P*
Cephalonia (n=2)	0.200	10.723 (0.587-195.918)	0.110
Chios (n=13)	0.318	19.164 (1.145-320.750)	0.040
Crete (n=4)	0.218	11.667 (0.671-202.757)	0.092
Frisarta (n=2)	0.625	67.452 (3.803-1,196.329)	0.004
Karagouniko (n=3)	0.233	12.785 (0.727-224.753)	0.082
Karystos (n=1)	0.000	reference	
Lesvos (n=5)	0.242	13.305 (0.776-228.228)	0.074
Vlahiko (n=3)	0.267	15.202 (0.869-265.926)	0.062

(b) Greek traditional indigenous breeds clustered together

Sheep breeds (no. of farms)	Prevalence	Odds ratio (95% CI)	*P*
Chios (n=13)	0.318	1.640 (1.142-2.355)	0.007
Greek traditional indigenous breeds (n=18)	0.221	reference	
Frisarta (n=2)	0.625	5.865 (2.950-11.660)	<0.001

(c) Imported breeds considered individually

Sheep breeds (no. of farms)	Prevalence	Odds ratio (95% CI)	*P*
Assaf (n=2)	0.100	Reference	
Lacaune (n=23)	0.280	1.554 (0.510-4.736)	0.439

Supplementary material 1. Detailed description of procedures and techniques employed in the study.

1. Sheep farms and animal sampling

In total, 111 sheep farms in the 13 administrative regions of Greece were included into the study and visited for collection of samples and information. Veterinarians active in small ruminant health management around Greece, were contacted and asked if they wished to collaborate in the investigation. In total, 23 veterinarians had agreed to collaborate. Farms were selected by the collaborating veterinarians on convenience basis (i.e., willingness of farmers to accept a visit by University personnel for sampling animals). The principal investigators (NGCV, GCF) visited all farms for sample collection. Farms were classified according to management system followed therein, as intensive (n=26), semi-intensive (n=57), semi-extensive or extensive (n=28), by following the criteria of the European Food Safety Authority (2014).

In each farm, 20 clinically healthy ewes (*secundiparae* or older) were selected at random for sampling. For selection of animals, farmers had been asked to remove *primiparae* ewes and ewes with known udder abnormalities from the main flock. A standardised clinical examination (observation, palpation, comparison between glands) of the udder was performed, always by the principal investigator (NGCV) (Fthenakis, 1994; Mavrogianni et al., 2005) and the first two squirts of secretion were drawn on the gloved hand of an assisting investigator and assessed. All investigators involved in sampling procedures wore disposable, non-sterile latex gloves. If udder abnormalities were recorded during clinical examination, the ewe was excluded from sampling. Animals found with abnormalities and excluded, were not replaced.

Standard methods for aseptic collection of milk samples were followed (Fthenakis, 1994). Then, 10 to 15 mL of secretion were collected into a sterile container; separate samples were collected from each mammary gland into separate containers. Milk samples were then drawn onto a paddle for performing the California Mastitis Test (CMT). For transportation, samples were stored into portable refrigerators with ice packs and transported by car; for samples collected in islands, airplane or boat transportation, as accompanying luggage, was also involved.

2. Paraclinical examinations

Laboratory procedures started within 24 h after collection. Milk samples (10 μL) were cultured using Columbia blood agar plates incubated aerobically at 37 °C for up to 72 h. Bacterial identifications were performed by using standards methods (Barrow and Feltham, 1993; Euzeby, 1997).

After sample collection, at ewe-side, all samples were tested by use of the CMT. The test was performed as previously described for ewes' milk (Fthenakis, 1995); it was carried out and scored always by the same person, i.e., the principal investigator (NGCV). Five degrees of reaction ('negative', 'trace', '1', '2', '3') were described (Schalm et al., 1971). Milk smears were also produced and dried. The milk smears were stained by the Giemsa method for estimation of leucocyte subpopulations; proportion of leucocyte types therein was calculated by observing at least 10 fields of each milk film under magnification 10×. Subsequently, the Microscopic cell counting

method (Mccm) (IDF reference method) (International Dairy Federation, 1984; Contreras et al., 2007; Raynal-Ljutovac et al., 2007) was performed in 894 samples (20.3% of all samples).

3. Data management and analysis

Ewes were considered to have subclinical mastitis when a bacteriologically positive milk sample ([a] >10 colonies of the same organism and [b] no more than two different types of colonies) with concurrently increased CMT score (≥'1') plus neutrophil and lymphocyte proportion (≥65% of all leucocytes) was detected (Fragkou et al., 2014). The definition referred to ewes (hence, animals with both glands affected were counted as one case).

Quantitative information on the cellular content of ewes' milk was obtained by using two sets of data: the CMT results and the results of the Mccm. Although it is generally established that CMT results are reliable proxy measurements for somatic cell counts (SCCs) (Fthenakis, 1995; Gonzalez-Rodríguez and Carmenes, 1996), we further confirmed that in the present study. Following assignment of numerical values to CMT scores (value 0 to score 'negative', value 1 to score 'trace', value 2 to score '1', value 3 to score '2', and value 4 to score '3') and $\log_{10}$-transformations, correlation between CMT scores and Mccm SCCs was r=0.913 (95% CI: 0.902-0.923) (P<0.001) and the corrected R^2 was 83.4%; significance of the difference between r and rho (the correlation hypothesized to exist within the population from which the sample had been drawn) was P<0.001.

For analysis, data were entered into Microsoft Excel and analysed using IBM SPSS Statistics (ver. 21) (IBM; Armonk, NY, USA). The outcome of 'subclinical mastitis' was considered. Exact binomial confidence intervals (C.I.) were obtained. A preliminary assessment of the importance of predictors was performed using by cross-tabulation with the chi-square test, and with simple logistic regression without random effects. Subsequently, mixed-effects logistic regression was employed to perform the same comparisons, using the different farms (n=111) as a 'random effect'. Then, analysis of variance was employed and the following comparisons were made between farms in relation to this outcome:

(a) farms with pure-bred animals *versus* farms with cross-bred animals,

(b) farms with Greek pure-bred animals *versus* farms with imported pure-bred animals,

(c) farms with imported pure-bred animals *versus* all other farms (i.e., farms with Greek pure-bred animals and farms with cross-bred animals),

(d) farms with the various Greek pure-bred animals (in total, 8 breeds), farms with imported pure-bred animals (in total, 2 breeds) and farms with cross-bred animals and

(e) farms with the various pure-bred animals (in total, 10 breeds) between them.

Subsequently, farms with the Greek breeds Cephalonia, Crete, Karagouniko, Karystos, Lesvos and Vlahiko were considered together in a cluster termed 'Greek traditional indigenous breeds' (n=18 farms), as initial comparison between those farms did not show significant difference. Then, comparisons between the various breeds were repeated with smaller number of breeds (in total, 3 Greek pure-breeds and 5 breeds in total).

Finally, a multivariable model was created using mixed-effects logistic regression with farm as the random effect, which included as variables the management system in farms and the sheep breed. The analysis was repeated by considering farms under semi-extensive and extensive management clustered together (i.e., using 3 categories in the management system).

Statistical significance was defined at ≤ 0.05.

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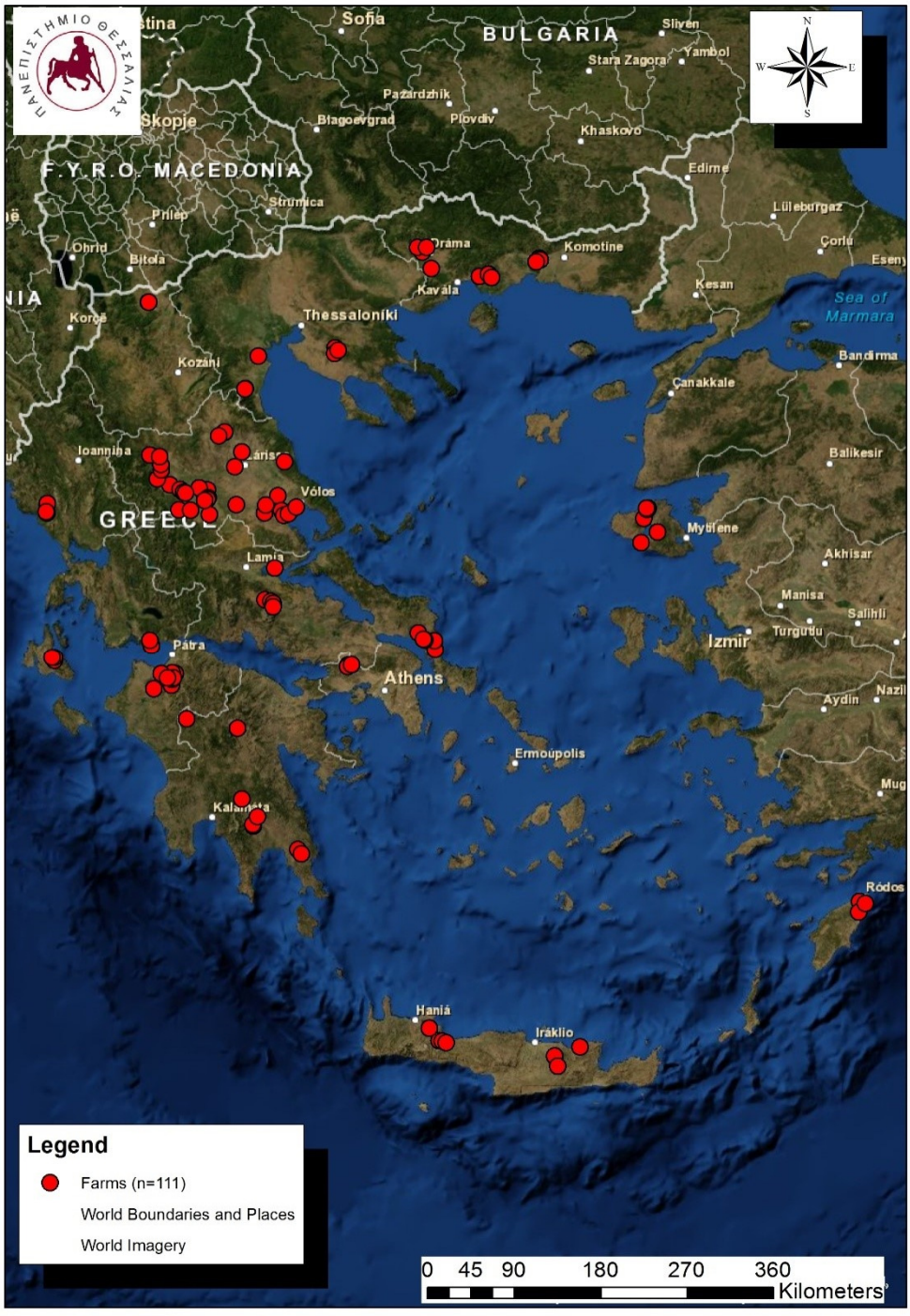
Supplementary material 2. Location of 111 farms included in the study around Greece.



Supplementary material 3. Breeds in sheep farms in Greece according to management system applied in farms.

Sheep breeds	Management system (no. of farms)			Total
	Intensive	Semi-intensive	Semi-extensive or extensive	
1. Pure-breeds	17	25	16	58
1.1. Greek breeds	6	13	14	33
1.1.1. Cephalonia		1	1	2
1.1.2. Chios	6	4	3	13
1.1.3. Crete			4	4
1.1.4. Frisarta		2		2
1.1.5. Karagouniko		2	1	3
1.1.6. Karystos			1	1
1.1.7. Lesvos		4	1	5
1.1.8. Vlahiko			3	3
1.2. Imported breeds	11	12	2	25
1.2.1. Assaf	1	1		2
1.2.2. Lacaune	10	11	2	23
2. Cross-breeds	9	32	12	53
Total	26	57	28	111