

A serosurvey for selected pathogens in Greek European wild boar

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ABSTRACT

Objectives: Serum samples, collected from 94 European wild boar (*Sus scrofa*) during the hunting seasons 2006–2010 from different regions of Greece, were examined in order to estimate the role of these wildlife species as reservoir of pathogens important for livestock and/or public health.

Materials and Methods: The assays used for this purpose were commercial indirect ELISA for the detection of antibodies against porcine circovirus type 2 (PCV-2), porcine reproductive and respiratory syndrome (virus) (PRRSV), Aujeszky's disease virus (ADV), influenza A (IA) virus, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Salmonella* species, *Trichinella* species and indirect immunofluorescence antibody test for the detection of antibodies against *Toxoplasma gondii* and *Neospora caninum*.

Results: Antibodies against PCV-2, PRRSV, ADV, IA virus, *A. pleuropneumoniae*, *M. hyopneumoniae*, *Salmonella* species, *Trichinella* species, *T. gondii* and *N. caninum* were detected in 19.1 per cent, 12.8 per cent, 35.1 per cent, 1.1 per cent, 57.4 per cent, 0 per cent, 4.3 per cent, 6.4 per cent, 5.2 per cent and 1.1 per cent of the samples, respectively. Cluster analysis revealed a hot spot of seropositivity near Bulgarian border; seropositivity to ADV was more common among female animals.

Conclusions: These results indicate exposure of wild boar to most of the above-mentioned pathogens, raising concern about the possibility that these species may pose a significant health risk for livestock and/or humans.

INTRODUCTION

In the last decades, an increase of wild boar population in Europe has been documented (Sáez-Royuela and Tellería 1986, Laddomada 2000). In Greece, wild boar is a native species with a wide distribution in all over the continental part of the country (Tsachalidis and Hadjisterkotis 2009). Recently, their population density has increased in many areas, especially those with shrub lands, agroforestry formations in

combination with cultivations (cereals) (Birtsas and others 2007, Giannakopoulos 2012). Wild boar is one of the most popular big-game species in Greece with an increasing interest from hunters and can be found in many habitat types.

As the number of European wild boar increases, the interaction with domestic livestock also increases, and this raises concerns of direct and indirect human exposure to zoonotic agents (Gortázar and others 2007). Wild boar may represent reservoir of a long list of viral, bacterial and parasitic agents and may play a direct or indirect role on the circulation, maintenance and transmission of infectious diseases to domestic pigs. For example, wild boar are considered a limiting factor for the eradication of infectious diseases with significant economic impact in swine industry such as Aujeszky's disease virus (ADV) or porcine circovirus type 2 (PCV-2) (Meng and others 2009). Furthermore, the interface of humans with boars via hunting or agricultural purposes or consumption of infected wild boar meat is the basic reason that creates the appropriate circumstances for the transmission of importance for the public health pathogens such as trichinellosis or toxoplasmosis (Meng and others 2009).

Control of wild boar diseases requires sound epidemiological information in order to investigate the prevalence of their exposure to various pathogens and to determine their geographical distribution in a country before the application of control measures (Morner and others 2002). Geographical information systems (GIS) represent a modern tool for surveillance of wildlife diseases and offer spatial analysis, which is an essential component of modern disease surveillance systems (Pfeiffer and Hugh-Jones 2002). GIS can be used to correlate environmental, climatic, socio-economic and many other data and factors with the prevalence of



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exposure to the infectious agent(s) in question. Although there are many surveys indicating the role of domestic pigs on the transmission of pathogens in Greece, to the best of the authors' knowledge, there is only sparse information on the role of European wild boar (Sofia and others 2008, Billinis 2009).

The aim of this study was to investigate the seroprevalence for 10 selected pathogens, important for livestock and/or public health, in wild boar from different areas of Greece and to correlate results with environmental factors within a GIS.

MATERIALS AND METHODS

In collaboration with the Hunting Federation of Macedonia and Thrace, blood samples were collected from 94 free-ranging European wild boar from different areas of Greece during the hunting seasons 2006–2010. Hunters collected the blood samples by heart puncture or from the body cavities, and they stored them into sterile tubes at 4°C until arrival to the laboratory, within 24 hours. Sera were separated after centrifugation and were stored at –20°C pending examination.

Sera were tested for antibodies against PCV-2 using a commercial indirect ELISA (Ingezim Circo IgG, Ingenasa, Madrid, Spain). Also, ELISAs were used for the detection of antibodies against porcine reproductive and respiratory system (PRRS) (Porcine Reproductive and Respiratory Syndrome Virus Antibody Test Kit, IDEXX Laboratories, Westbrook, USA), ADV (PRV/ADV Gb Ab Test, IDEXX laboratories, Westbrook, USA), IA viruses (Ingezim Influenza Porcina, Ingenasa, Madrid, Spain), *Actinobacillus pleuropneumoniae* (APP-Apx IV Ab Test, IDEXX laboratories, Westbrook, USA) and *Mycoplasma hyopneumoniae* (M. hyo Ab Test, IDEXX, Westbrook, USA). Detection of antibodies against *Salmonella* species was done by an ELISA (Swine Salmonella Ab Test, IDEXX, Westbrook, USA), which detects antibodies against a broad range of *Salmonella* serogroups. Antibodies against *Trichinella* species were determined using a commercial kit (ELISA *Trichinella* Serum Screening, Institut Pourquier, Montpellier, France) that has been validated for wild boar, and it is based on the excretory/secretory antigen of the parasite. All the above ELISAs were performed following the manufacturers' recommendations.

Finally, anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies were detected by indirect fluorescence antibody test kits using commercially available slides coated with parasite tachyzoites (Fuller Laboratories, Fullerton, California, USA) and anti-porcine IgG conjugate (Porcine IgG FITC conjugate, VMRD Inc) was used. Serum samples were tested at twofold dilutions in PBS, starting from 1:40 (cut-off titre) until reaching the end-point titre.

The area from where all the 94 samples were obtained was located in the field using handheld Global Positioning System units or using longitude and latitude information

provided by the hunters on Google Earth software (<https://earth.google.com/>). GIS layers were created to represent the geographic locations of the wild boar serum samples and of the free-ranging swine farms. The environmental variables for this study were derived from two main database categories: altitude and land cover. Altitude was extracted from a digital elevation model with a spatial resolution of 1 km² (<http://srtm.csi.cgiar.org/Index.asp>) and land use were derived from the Corine Land Cover 2006 database (European Environment Agency, www.eea.europa.eu/data-and-maps). These data sets were converted to a common projection (Greek Grid projection system), map extent and resolution prior to use. ArcGIS V.10.1 GIS software (ESRI, Redlands, California, USA) was employed for description and analysis of spatial information. Cluster analysis for the seropositivity to at least one of the examined pathogens was performed with the Hot Spot Analysis tool that calculates the Getis-Ord Gi* statistic (Mitchell 2005).

Data on wild boar population density in each regional unit were gathered through a questionnaire survey of local Game offices of Forest services, Federal Rangers and members of local hunting clubs. An index with six classes of population density was created using the data of this survey. Moreover, the authors also carried out 112 interviews (76 federal rangers, 6 scientific collaborators of the Hunting Federation of Macedonia and Thrace, 20 heads of wild boar hunters and members of local hunting clubs and 10 local Game offices of Forest services). Interviews were targeted to determine current wild boar presence and the estimated local population size. Reported data were plotted on Google Earth software.

The relationship between wild boar sex and seropositivity to each pathogen was examined with the Phi coefficient (Cheetham and Hazel 1969).

The authors examined the relationship between seropositivity to each particular pathogen and selected environmental variables (altitude, distance from the nearest free-ranging swine farms, land use, land cover) and the density of wild boar population. Because the first two variables were continuous, the hypothesis was tested with independent samples *t* test or, whenever the counts of seropositive or seronegative animals were less than five, with the non-parametric equivalent Mann-Whitney U test (Bradley 2007). The latter test was also used to check for possible relationship between seropositivity and wild boar population density; in addition, the authors used the Kendal tau correlation measure, which is suitable for comparing two categorical variables. Considering the environmental variables land use and land cover, the authors used the uncertainty coefficient, which is a measure for testing the relationships between two nominal variables, when one of them is considered a dependent variable (Fowler and others 2013). The analysis was performed with IBM SPSS V.22.0 (Gray and Kinnear 2012), and the results were considered significant when $P \leq 0.05$. The authors also used the Cramer's

V measure in order to compare the seroprevalences between the mountain ranges A, B and C.

RESULTS

The number of positive samples for each pathogen and distance between seropositive animals and closest free-ranging swine farm are shown in Table 1. The locations where viral pathogen-seropositive wild boar samples have been collected are presented in Fig 1, while the same information for bacterial and parasitic pathogens is shown in Fig 2. Among the various pathogens examined, seropositivity was more common against *A. pleuropneumoniae*, followed by ADV, PCV-2 and PRRS virus, while antibodies against the remaining agents examined were detected in <10 per cent of the samples. None of the sera tested was positive for antibodies against *M. hyopneumoniae* (analytical results per sample in online supplementary file 1).

Figure 3 shows the origin of seropositive and seronegative wild boar samples included in the present study. The Gi* statistic for each feature in the data set appears as a z-score. The latter revealed a cluster (hot spot) of seropositivity to at least one of the pathogens examined near the Bulgarian borders (see online supplementary fig S1).

The mean altitude where seropositive wild boar samples have been obtained was 839.5 m above sea level (range 175–1720 m; sd 334.94 m). Most of them have been collected in tree cover, broadleaved, deciduous, closed forests, followed by shrub cover, closed-open, evergreen forests and fewer seropositive samples have been collected in agroforestry formations and cultivated land. Moreover, 855 free-ranging swine farms were present in the areas of the country inhabited by wild boar.

The seropositive wild boar have been hunted in regional units with medium or high wild boar population density according to the GIS population index layer (Fig 4). The highest mean density of hunted animals was observed in the Ioannina regional unit (1.5 ind/km²) and the lowest in the Grevena regional unit (0.17 ind/km²).

Although sex was known for only 51 out of the 94 wild boars, there was a slightly significant relationship with seropositivity to ADV (p=0.046), which was higher in females; 52.6 per cent of the females were seropositive versus 25 per cent among males.

There were no significant relationships among environmental factors (altitude, distance from the nearest free-ranging swine farms, land use, land cover) or density of wild boar population and seropositivity with the only exception of PRRS virus where a borderline association with land cover (P=0.05) was found (higher seropositivity in cultivated and managed areas). The above relationships were not apparent when the authors used the Bonferroni correction due to multiple testing, but they note them as indications of a possible interdependence. Also, Cramer's V measure was significant,

TABLE 1: Seroprevalence for 10 selected pathogens in European wild boar from Greece

Pathogen	Serological assay	Seropositivity (%; 95% CI)			
		Overall (n=94)	Mountain range A Rodopi (n=43)	Mountain range B CENTAL (n=28)	Mountain range C Pindos (n=23)
Porcine circovirus type 2 (PCV-2)	ELISA (Ingenasa)	18/94 (19.1%; 11.15 to 27.05)	5/43 (11.6%; 2.03 to 21.17)	9/28 (32.1%; 14.81 to 49.39)	4/23 (17.4%; 1.91 to 32.89)
Porcine reproductive and respiratory syndrome virus (PRRSV)	ELISA (IDEXX laboratories)	12/94 (12.8%; 6.05 to 19.55)	3/43 (7%; -0.63 to 14.63)	9/28 (32.1%; 14.81 to 49.39)	0/23 (0%)
Aujeszky's disease virus (ADV)	ELISA (IDEXX Laboratories)	33/94 (35.1%; 25.45 to 44.75)	16/43 (37.2%; 22.75 to 51.65)	14/28 (50%; 31.48 to 68.52)	3/23 (13%; -0.74 to 26.74)
Influenza A (IA) virus	ELISA (Ingenasa)	1/94 (1.1%; -1.01 to 3.21)	1/43 (2.3%; -2.18 to 6.78)	0/28 (0%)	0/23 (0%)
<i>Actinobacillus pleuropneumoniae</i>	ELISA (IDEXX Laboratories)	54/94 (57.4%; 47.4 to 67.4)	22/43 (51.2%; 36.26 to 66.14)	18/28 (64.3%; 46.55 to 82.05)	14/23 (60.9%; 40.96 to 80.84)
<i>Mycoplasma hyopneumoniae</i>	ELISA (IDEXX Laboratories)	0/94 (0%)	0/43 (0%)	0/28 (0%)	0/23 (0%)
<i>Salmonella</i> species	ELISA (IDEXX Laboratories)	4/94 (4.3%; 0.2 to 8.4)	1/43 (2.3%; -2.18 to 6.78)	1/28 (3.6%; -3.3 to 10.5)	2/23 (8.7%; -2.82 to 20.22)
<i>Trichinella</i> species	ELISA (Institut Pourquier)	6/94 (6.4%; 1.45 to 11.35)	2/43 (4.6%; -1.66 to 10.86)	1/28 (3.6%; -3.3 to 10.5)	3/23 (13%; -0.74 to 26.74)
<i>Toxoplasma gondii</i>	Indirect fluorescent antibody test (Fuller Laboratories)	5/94 (5.2%; 0.71 to 9.69)	4/43 (9.3%; 0.62 to 17.98)	0/23 (0%)	1/23 (4.3%; -3.99 to 12.59)
<i>Neospora caninum</i>	Indirect fluorescent antibody test (Fuller Laboratories)	1/94 (1.1%; 1.01 to 3.21)	0/43 (0%)	1/28 (3.6%; -3.3 to 10.5)	0/23 (0%)

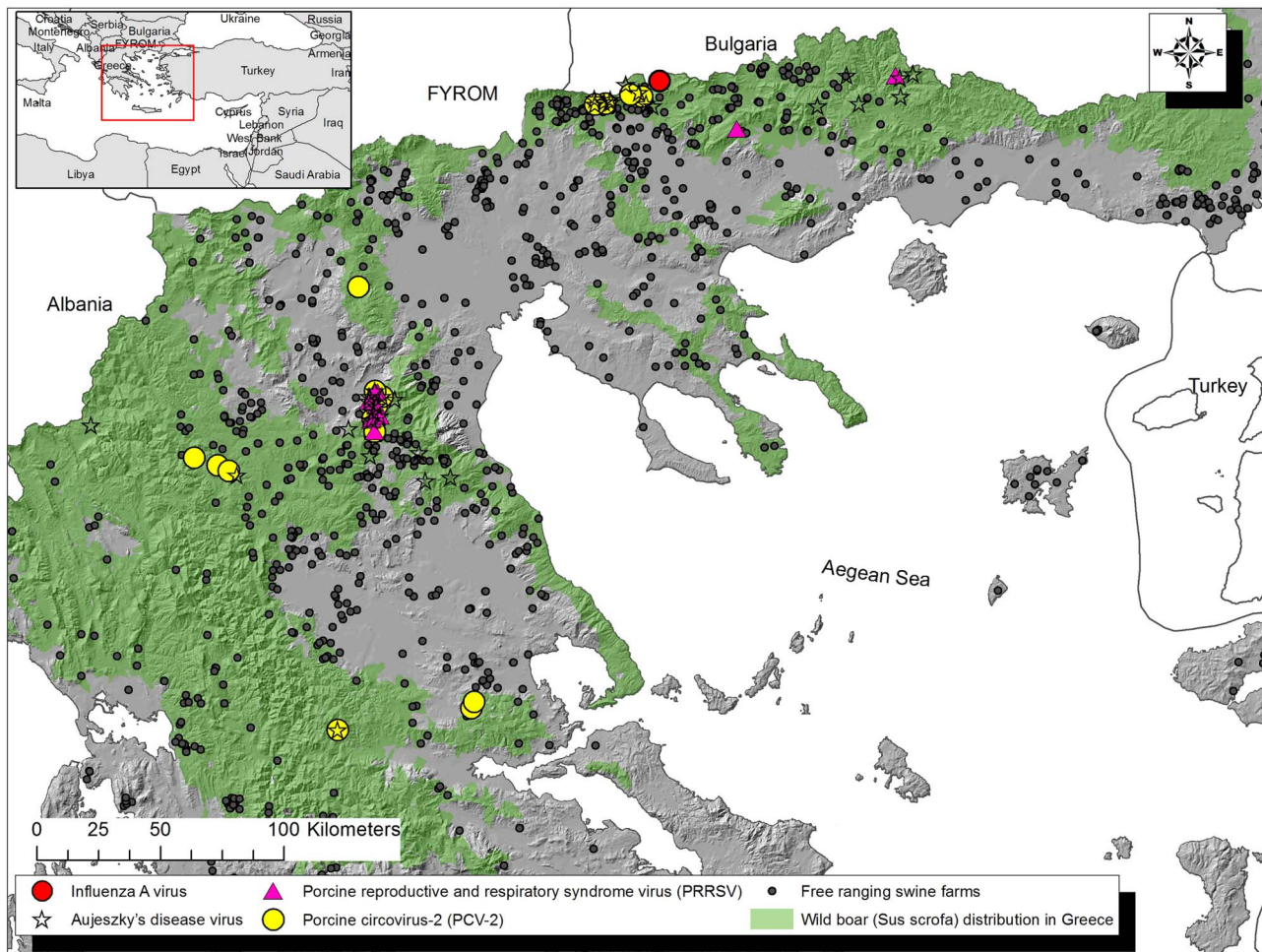


FIG 1: Map of the study area, showing the origin of wild boar samples found seropositive to various viral agents. Wild boar distribution in the area and the presence of free-ranging swine farms are also demonstrated (according to Tsachalidis and Hadjisterkotis 2009, Giannakopoulos 2012)

taking into account the Bonferroni correction, in two cases: PRRS (Cramer's $V=0.387$, $P=0.001$) and ADV (Cramer's $V=0.287$, $P=0.021$). In both cases, seropositivity rate was higher in the mountain range B. Considering PRRS, seropositivity rates in the mountain ranges A, B and C were 7 per cent, 32.1 per cent and 0 per cent, respectively, whereas the corresponding figures for ADV were 37.2 per cent, 50 per cent and 13 per cent, respectively.

DISCUSSION

This is the first expanding serological survey of Greek wild boar for antibodies against pathogens with economic and/or zoonotic importance. The results of this study highlight the possible role in the circulation of viral, bacterial and parasitic pathogens in Greece. The high seropositivity rates for PCV-2 (19.1 per cent), ADV (35.1 per cent) and *A. pleuropneumoniae* (57.4 per cent) are compatible with data from other European countries (Gortázar and others 2002, Vicente and others 2002, 2004, Ruiz-Fons and others 2006, Vengust and others 2006, Sedlak and others 2008, Montagnaro and others

2010, Boadella and others 2012b, Roic and others 2012). On the contrary, the detection of antibodies against PRRS virus in 12.8 per cent of the samples was rather unexpected as many surveys have shown that the seroprevalence rates in free-ranging wild boar are usually low to nil in contrast to farmed or semi-captive wild boar (Albina and others 2000, Vicente and others 2002, Vengust and others 2006, Montagnaro and others 2010, Boadella and others 2012b, Roic and others 2012). Although seropositivity to PRRS virus may indicate contact between wild boars and domestic pigs, the intensive piggeries in Greece is not reachable to wild animals. Nevertheless, the latter is possible between wild boar and semi-captive pigs, taking into account that PRRS virus can be transmitted up to 4.7 km away from the source of contamination (Dee and others 2009).

There are indications that wild boar can play a role in the epidemiology of IA viruses, as antibodies against the three subtypes of swine influenza virus (SIV), H1N1, H3N2 and H1N2, have been detected in free and semi-captive wild boar (Ruiz-Fons and others 2008). In this study, seroprevalence was 1.1 per cent, whereas on a global level varies from 0 per cent to 75 per cent (Saliki

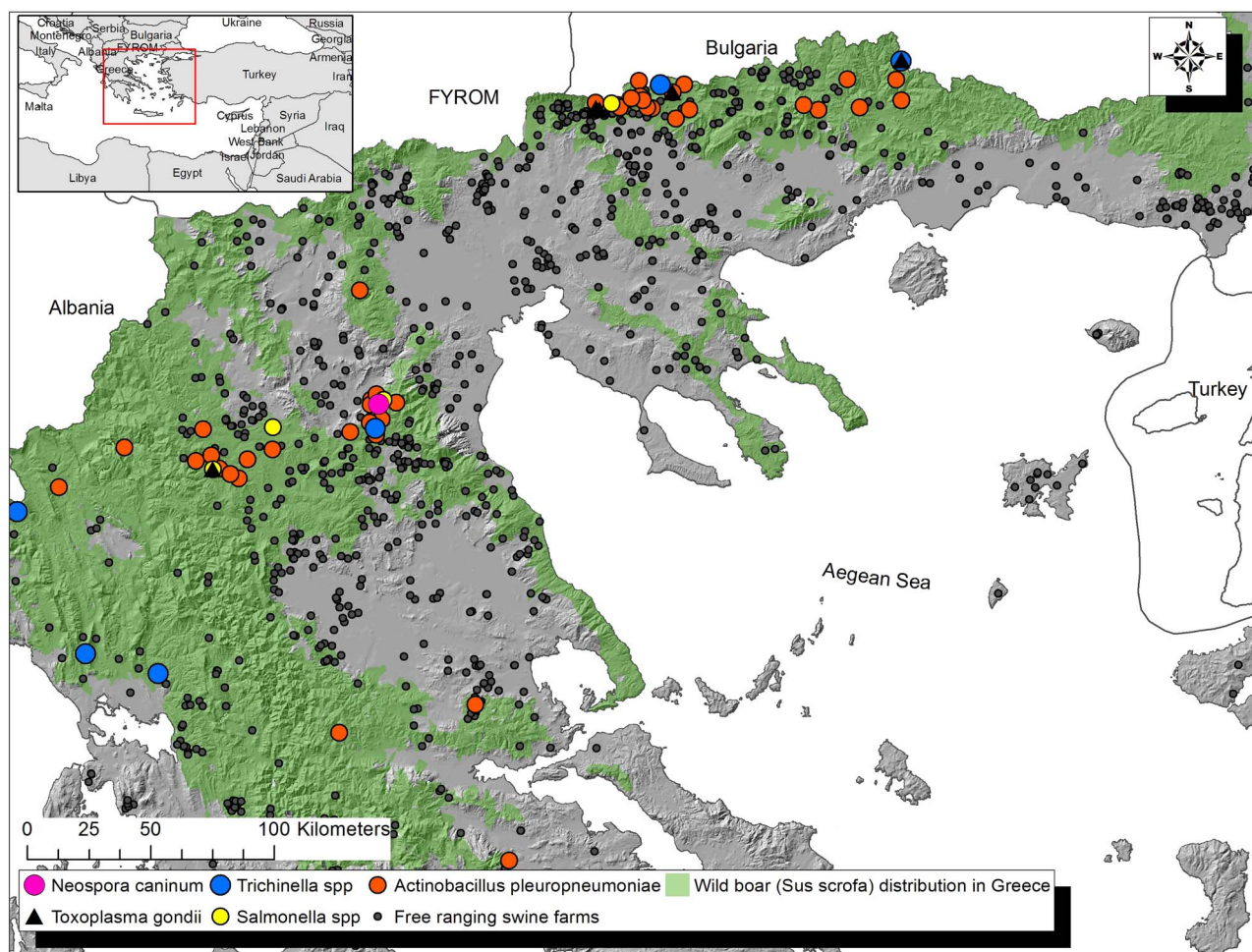


FIG 2: Map of the study area, showing the origin of wild boar samples found seropositive to various bacterial and parasitic agents. Wild boar distribution in the area and the presence of free-ranging swine farms are also demonstrated (according to Tschalidis and Hadjisterkotis 2009, Giannakopoulos 2012)

and others 1998, Gipson and others 1999, Vicente and others 2002, Vengust and others 2006, Kaden and others 2008, Sattler and others 2012). Every time that a high seroprevalence has been recorded, it applied to semi-captive or farmed animals, living at high density, which is a prerequisite for SIV to become endemic.

Antibodies against *Salmonella* species were detected in 4.3 per cent of the samples, which is compatible with the results from Spain (Vicente and others 2002). On the other hand, in some countries such as Italy (Montagnaro and others 2010) and Slovenia (Vengust and others 2006), seropositivity rates have been much higher (19.3 per cent and 47 per cent, respectively).

In countries where the density of wild boar is low and pig industry follows modern hygiene standards, *Trichinella* species transmission cycle is blocked (Poizio 2007). Although no human cases had been reported for decades in Greece (Sotiraki and others 2001), a medium (6.4 per cent) seropositivity rate was found in the present study compared with other European countries where seropositivity ranges from 0.11 per cent to 14 per cent (Poizio 2007, Hurníková and Dubinský 2009,

Richomme and others 2010). Since health management is not applicable to wild boars, the seropositivity in this population may be of particular importance for the surveillance for this zoonotic agent, although the serological tests in free-ranging wild boar may overestimate its prevalence (Boadella and others 2012a).

Anti-*T. gondii* antibodies were found in 5.2 per cent of the samples, whereas the relevant figures in previous studies conducted in different European countries were 26.2 per cent for Czech Republic (Bártová and others 2006), 36.3 per cent for Spain (Ruiz-Fons and others 2006), 8.1 per cent for Slovak Republic (Antolová and others 2007), 23 per cent for France (Beral and others 2012) and 20.6 per cent for Portugal (Coelho and others 2014). Although the serological techniques used in the above studies, except one (Bártová and others 2006), differ from this study and thus a direct comparison of prevalences is not possible, these figures may be helpful to highlight the differences in the serological status of wild boar population against *T. gondii* across European countries. Gauss and others (2005) support that when wild boars live in restricted areas and at high

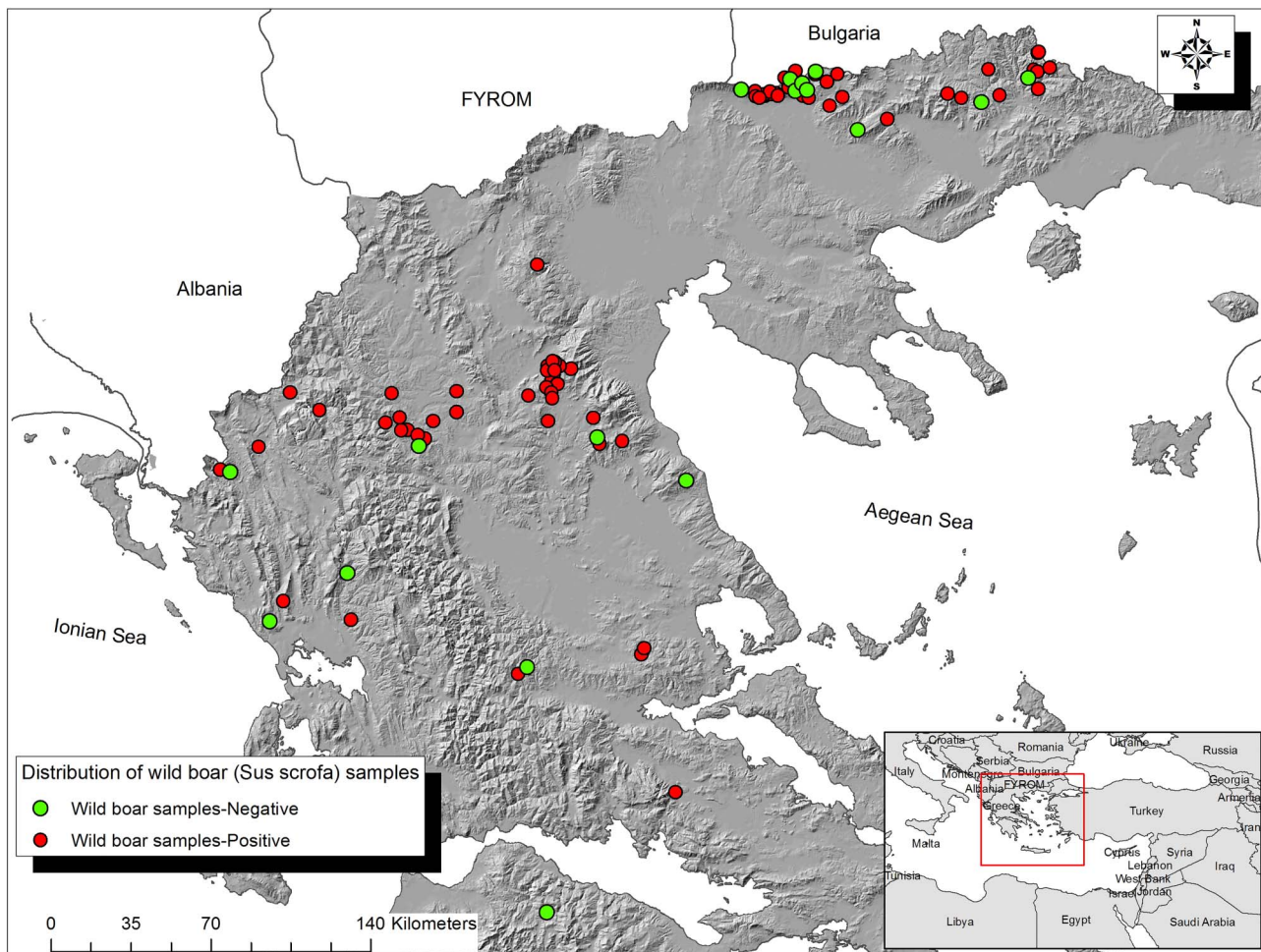


FIG 3: Map of the study area, showing the origin of wild boar samples found seropositive to at least one of the 10 pathogens (red dots) and those found seronegative to all of the 10 pathogens (green dots)

density they have an increased possibility of contact with oocysts.

The GIS analysis of the present study demonstrated that 855 from a total of >2000 free-ranging swine farms currently present in Greece are located inside the wild boar's distribution range, and this may represent an underestimate since there are some smaller farms inside the study area that may have not recorded. Home range size for wild boar varies from 4 to 31 km², but they can move up to 6 km outside this area (Mailard and Fournier 1995, Sodeikat and Pohlmeier 2007, Gaston and others 2008). Therefore, this study's data indicate that there is a substantial risk of mutual transmission of pathogens between the wild boar and the free-ranging swine populations.

Statistical analysis of the data showed that female wild boar were more frequently seropositive for ADV than the males. This female predisposition is similar with that reported in previous studies and has been attributed to the increased intraspecific contacts of females due to the younger age they reach sexual maturity and their social behaviour (Vicente and others 2005, Cano-Manuel and others 2014). The borderline

association between the high PRRS seropositivity rates and the cultivated and managed areas, where land is basically used for agricultural purposes, may be attributed to wild boar invading such areas searching for food. The human activity in combination with the presence of domestic pig farms in these areas creates suitable circumstances for the spreading of the PRRS virus to wild boar as the virus can be transmitted not only via direct but also via indirect routes such as vehicles, people involved in the swine industry and flying insects.

The recent rapid demographic expansion of wild boar (*Sus scrofa*) all over Europe, including Greece, may influence the epidemiology of various pathogens that can affect not only livestock but also humans and are transmitted and spread by various routes. Based on hunting bag estimates, the number of wild boar in Greece is increasing year by year and during the last hunting season it exceed 25,000 individuals. Although limited, the evidence of exposure of wild boar to IA virus, *Salmonella* species, *Trichinella* species and *T. gondii*, which are some of the most important zoonotic pathogens worldwide, indicates that this animal species may be important for their spread and their

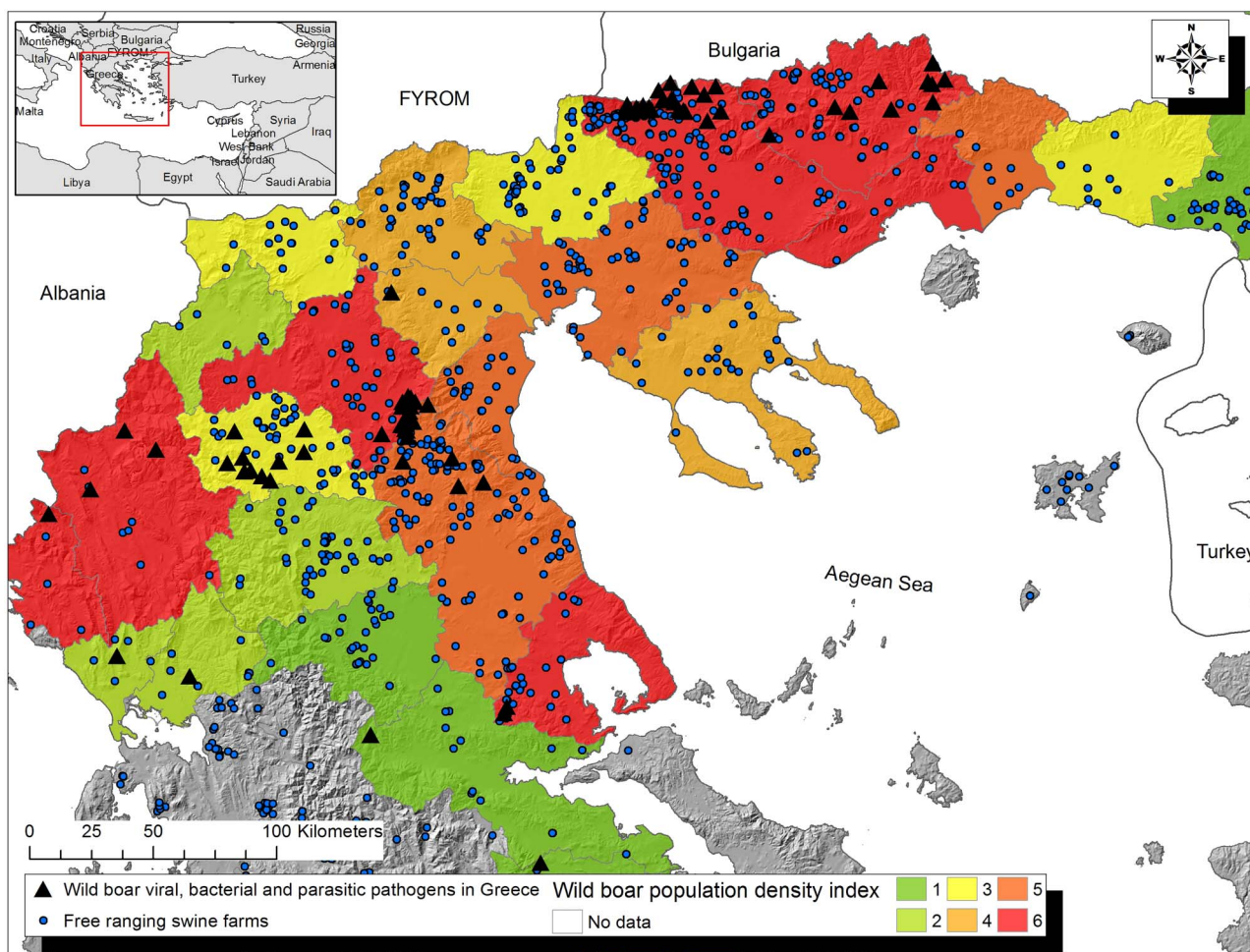


FIG 4: Map of the study area, showing the origin of wild boar samples found seropositive to at least one of the 10 pathogens, the free-ranging swine farms and the overall wild boar population density

maintenance in the environment and may represent a health risk for people handling or consuming them. In addition, the unclear situation of the wild boar's infectious status in bordering countries and the possible spread of infectious agents because of their migration across Greek borders make the regular monitoring of wild boar diseases essential. Furthermore, the role of wild boar as a source of infectious agents for other species should be extensively studied to determine their impact on swine industry.

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