



# The Balkans and the colonization of Europe: the post-glacial range expansion of the wild boar, *Sus scrofa*

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## ABSTRACT

**Aim** We focus on the biogeographical role of the Balkan Peninsula as a glacial refugium and source of northward post-glacial dispersal for many European taxa. Specifically, we analysed the genetic structure and variation of wild boar (*Sus scrofa*) samples primarily from Greece, a region that has repeatedly served as a glacial refugium within the Balkan Peninsula.

**Location** Continental Greece, the Aegean island of Samos and Bulgaria.

**Methods** We analysed wild boar samples from 18 localities. Samples from common domestic breeds were also examined to take into account interactions between wild and domesticated animals. Phylogenetic analyses were carried out on a 637-bp fragment of the mitochondrial DNA control region in 200 wild boar and 27 domestic pigs. The sequences were also compared with 791 Eurasian wild boar and domestic pig D-loop sequences obtained from GenBank.

**Results** Ninety-four haplotypes were identified in the European wild boar data set, of which 68 were found in the Balkan samples and assigned to two previously described clades: the E1 European and Near Eastern clades. All of the continental samples clustered in the E1 clade and the samples from Samos fell into the Near Eastern clade, consistent with the island's proximity to Asia Minor. Intriguingly, 62 novel haplotypes were identified and are found exclusively in the Balkans. Only six haplotypes were shared between wild boar and domestic pigs.

**Main conclusions** Our data reveal numerous novel and geographically restricted haplotypes in wild boar populations, suggesting the presence of separate refugia in the Balkans. Our analyses support the hypothesis of a post-glacial wild boar expansion consistent with the leading edge model, north and west from modern day Greece, and suggest little maternal introgression of Near Eastern and domestic haplotypes into wild Balkan populations.

## Keywords

Balkan Peninsula, European wild boar, mitochondrial DNA, phylogeography, post-glacial colonization, *Sus scrofa*.

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## INTRODUCTION

The present-day geographical distribution of many European temperate species has been shaped by Quaternary climatic fluctuations and ice ages (Hewitt, 2000). The typical scenario predicts allopatric divergence following population fragmentation into isolated geographical refugia during glacial periods (Avice *et al.*, 1998). Several lines of evidence suggest that populations of European temperate species went extinct in the

northern regions of Europe and survived in southern areas that served as refugia during the Last Glacial Maximum (LGM), followed by northward range expansions back into Europe during the interglacial warmer periods (Hewitt, 1999). The southern European peninsulas of Iberia, Italy and the Balkans (and also Turkey), as well as smaller regions such as the Carpathians and southern France, played host to those ice age refugia, and the populations in them eventually recolonized Europe, although there was probably an important inflow

from eastern regions near the Caspian Sea during the recolonization process (Sommer & Benecke, 2005; Sommer & Nadachowski, 2006; Schmitt, 2007; Sommer *et al.*, 2008, 2009; Sommer & Zachos, 2009). The populations that survived in these refugia usually had no or limited gene flow and evolved independently, resulting in separate genetic groups involved in the recolonization processes (Hewitt, 1996, 2000; Taberlet *et al.*, 1998). This hypothesis of refuge-specific genetic polymorphism has been used to reconstruct recent post-glacial range expansion routes in many animal taxa, including grasshoppers (Hewitt, 1999, 2000), hedgehogs (Berggren *et al.*, 2005), hares (Kasapidis *et al.*, 2005; Stamatis *et al.*, 2009), field mice (Michaux *et al.*, 2004), roe deer (Randi *et al.*, 2004) and red deer (Skog *et al.*, 2009).

Among the Mediterranean peninsulas, the Balkans have been postulated to be the primary source of post-glacial expansions because the Alps and the Pyrenees acted as migration barriers for northward movements of species from the other European refugia (Hewitt, 2000). Additionally, the Balkans served as a bridge for the movements of species that had been restricted to Asia Minor during Pleistocene glaciations (Hewitt, 1999) because the Balkan region of Europe and Asia Minor were connected by a land bridge during the last glaciation (Tzedakis *et al.*, 1997; Aksu *et al.*, 1999).

Quaternary climate change has been postulated to have shaped geographical patterns of genetic diversity in European wild boar (*Sus scrofa* Linnaeus, 1758), resulting in two major Old World continental clades – one in Asia and the other in Europe (Giuffra *et al.*, 2000; Okumura *et al.*, 2001; Alves *et al.*, 2003; Larson *et al.*, 2005). In Europe, Scandura *et al.* (2008, 2011a) proposed that the modern genetic structure of *S. scrofa* is the result of a post-glacial colonization from one or more southern refugia, including Iberia and the Balkans, but excluding the Italian Peninsula where a highly divergent mitochondrial DNA (mtDNA) clade is indigenous. However, indigenous patterns of genetic variation could have been eroded by the domestication of pigs and human migration across the Near East and Europe (Jones, 1998; Giuffra *et al.*, 2000; Larson *et al.*, 2005, 2007).

We ascertained the genetic structure of the southern Balkan wild boar population in an effort to understand the historical effect of this region as a glacial refugium, and to identify attributes of recolonization during the post-glacial expansion of this species. The identification of the ancient and recent processes that have contributed to the present genetic structure of wild boar could provide a basis for understanding the role of the Balkans in shaping the historical phylogeography of other European species.

## MATERIALS AND METHODS

### Sample collection and DNA extraction

A total of 200 wild boar samples from 18 locations in Greece and one location in Bulgaria were collected and analysed (Table 1, Fig. 1). Additionally, 27 domesticated pig samples

from five commercial breeds (Large White, Landrace, Pietrain, Duroc and Hampshire) were examined for comparison purposes. Muscle and/or liver tissue material was collected upon capture and stored in ethanol. Whole genomic DNA was extracted according to a cetyl trimethyl ammonium bromide (CTAB) protocol (Hillis *et al.*, 1996).

### PCR amplification and DNA sequencing

A portion (638 bp) of the mtDNA control (D-loop) region was amplified using the primers H16108 and L15433 (Watanobe *et al.*, 2001). This fragment corresponds to positions 15451–16088 of the reference mtDNA genome (Ursing & Arnason, 1998, accession number: AJ002189). The polymerase chain reaction (PCR) volume was 25 µL and contained 50 ng of DNA template, 1 unit of Qiagen *Taq* DNA polymerase (Qiagen Inc., Valencia, CA, USA), 2.5 µL of 10× reaction buffer, 2.5 mM MgCl<sub>2</sub>, 0.25 mM of each deoxynucleotide triphosphate (dNTP) and 12.5 pmol of each primer. The amplification was carried out in an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) and involved one initial denaturation step at 94 °C for 5 min followed by 33 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min 30 s and a single final extension step of 72 °C for 5 min. The PCR products were then purified using the Nucleospin Extra kit (Macherey-Nagel, Düren, Germany) and Sanger sequencing was performed by Macrogen Inc. (Seoul, Korea) using an ABI Prism 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

### Haplotype identification – genetic relationships of the mtDNA haplotypes

An alignment was created using the 227 sequenced individuals together with an additional 791 mtDNA control region sequences obtained from GenBank. These sequences consist of 307 wild samples (93 Asian and 214 European), 450 domestic and 34 feral samples from different locations worldwide (Ursing & Arnason, 1998; Watanobe *et al.*, 1999, 2001; Kijas & Andersson, 2001; Okumura *et al.*, 2001; Alves *et al.*, 2003, 2010; Gongora *et al.*, 2003, 2004; Larson *et al.*, 2005, 2007; Fang & Andersson, 2006; Fang *et al.*, 2006) (for GenBank accession numbers see Appendix S1 in Supporting Information). Sequence alignment was performed using the CLUSTALW 2.1 algorithm (Thomson *et al.*, 1994). Parameters taken into consideration for multiple alignment were gap opening = 15.00 and gap extension = 6.66. The number of distinct haplotypes in the sequence data set was computed using the DNASP 5 software (Librado & Rozas, 2009). Haplotype nucleotide diversity and mean number of pairwise nucleotide differences were also calculated with DNASP.

In order to examine the genetic relationships between the identified haplotypes a second data set was assembled by retaining only one sequence per haplotype. An alignment was constructed using the parameters described above, and the best fit model of nucleotide substitution was estimated using a

**Table 1** Genetic variability observed in mitochondrial DNA control region sequences in wild boar from 18 different Greek regions, European wild boar and Greek domestic pigs.

	Region	<i>n</i>	<i>T</i>	<i>Hp</i>	<i>V</i> (%)	<i>W</i> (%)	<i>PM</i>	<i>h</i> (± SD)	$\pi_n$
1.	Aitolokarnania (AI)	13	5	3	38.46	23.08	9	0.423	0.003
2.	Evrytania (EV)	12	3	2	25.00	16.67	3	0.318	0.002
3.	Fokida (FK)	11	5	3	45.45	27.27	4	0.455	0.002
4.	Fthiotida (FT)	14	3	2	21.42	14.29	5	0.495	0.002
5.	Voiotia (VO)	13	4	3	30.80	23.08	5	0.282	0.001
6.	Larisa (LR)	12	4	2	33.33	16.67	6	0.439	0.002
7.	Magnisia (MG)	6	3	2	50.00	33.33	9	0.600	0.004
8.	Trikala (TR)	4	2	0	50.00	–	2	0.500	0.002
9.	Ioannina (IO)	13	5	4	38.46	30.76	9	0.894	0.004
	Central Greece	98	24	19	24.49	19.39	17	0.599 (± 0.063)	0.001 (± 0.0003)
10.	Pella (PL)	6	6	4	100.00	66.67	10	1.000	0.006
11.	Thessaloniki (TH)	5	3	2	60.00	40.00	6	0.700	0.004
12.	Halkidiki (HL)	9	4	3	44.44	33.33	4	0.417	0.001
13.	Drama (DR)	11	6	3	54.55	27.27	4	0.711	0.003
14.	Kavala (KB)	16	13	10	81.25	62.50	15	0.876	0.005
15.	Rodopi (RO)	13	7	2	53.85	15.38	7	0.731	0.028
16.	Evros (ER)	18	13	10	72.22	55.56	7	0.686	0.002
	Northern Greece	78	39	25	50.00	32.05	24	0.948 (± 0.020)	0.004 (± 0.0003)
17.	Peloponnesus (PN)	7	6	2	85.71	28.57	7	0.857	0.003
18.	Samos (SM)	13	4	2	30.77	30.77	14	0.295	0.004
	Wild boar Greece	196	66	58	33.67	29.59	45	0.886 (± 0.020)	0.005 (± 0.0004)
19.	Bulgaria (BL)	4	3	1	75.00	25.00	6	0.833	0.005
	Wild boar Balkans*	205	68	62	33.17	30.24	47	0.892 (± 0.092)	0.011 (± 0.006)
	Wild boar Europe (no Balkans)*†	196	33	22	16.84	11.22	33	0.947 (± 0.010)	0.005 (± 0.0030)
	Wild boar Europe Overall*†	401	94	–	23.44	–	57	0.930 (± 0.012)	0.004 (± 0.0002)
20.	Domestic pigs (Greece)	27	15	9	55.55	33.33	25	0.849 (± 0.042)	0.013 (± 0.0007)

*n*, sample size; *T*, number of different haplotypes; *Hp*, population/region specific haplotypes; *V*, the percentage of the total number of haplotypes divided by the number of individuals; *W*, the percentage of the population-specific haplotypes divided by the number of individuals; *PM*, number of polymorphic sites; *h*, haplotype diversity (± SD, standard deviation);  $\pi_n$ , nucleotide diversity.

\*Including samples from GenBank (BK1 and BK2, for references refer to Materials and Methods).

†Wild boar belonging to the E1 clade.

hierarchical likelihood ratio test (hLRT) as implemented in MODELTEST 3.7 (Posada & Crandall, 1998). The parameters of the best fit model were included as priors for MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003) and eight Markov chain Monte Carlo (MCMC) searches were run for 30 million generations. Desert warthog (*Phacochoerus aethiopicus*) D-loop sequences were included as outgroups (GenBank accession number: AB046876). For each MCMC, a tree was sampled every 100 generations. The first 10% of the trees were discarded as burn-in. A consensus tree from the retained trees was computed with the PAUP\* 4.0 software for every MCMC (Swofford, 2003).

In order to further elucidate the relationships between closely related European haplotypes, a median-joining network (Bandelt *et al.*, 1999) approach was used. The network was constructed using only the European samples with the software NETWORK 4.5.1.6 (Fluxus Technology, <http://www.fluxus-engineering.com>) assuming equal weights for all mutations and setting the genetic distance parameter  $\epsilon$  to zero in order to restrict the choice of feasible links in the final network.

To evaluate the likelihood of recent events of population expansion and decline, mismatch distribution analysis and

Tajima's *D* test of neutrality (Tajima, 1989) were employed on the haplotypes that were found among the Balkan wild boar populations. Theoretical studies have demonstrated that populations in long, stable demographic equilibrium display a chaotic mismatch distribution, while recent rapid population expansions or bottlenecks are represented by a unimodal profile (Rogers & Harpending, 1992). Mismatch distributions for each population were established and their fit to Poisson distributions was assessed by 1000 Monte Carlo random simulations. Tajima's *D* neutrality test is widely used to test for population growth. A population experiencing expansion would result in a rejection of the null hypothesis of neutrality (i.e. give significant negative values). Estimation and testing of Tajima's *D* values and mismatch distribution analysis were performed using ARLEQUIN 3.11 (Excoffier *et al.*, 2005).

## RESULTS

A total of 245 haplotypes were identified in the data set, comprising both wild boar and domestic pigs. Of these, 102 clustered within the general European clade E1 (of which 94



**Figure 1** Map of the Balkans showing the distribution of wild boar sampling sites (dark circles) with site abbreviations as given in Table 1. Stars represent samples for which sequences were retrieved from GenBank (Larson *et al.*, 2005, 2007). The dotted line divides continental Greece into northern and central parts.

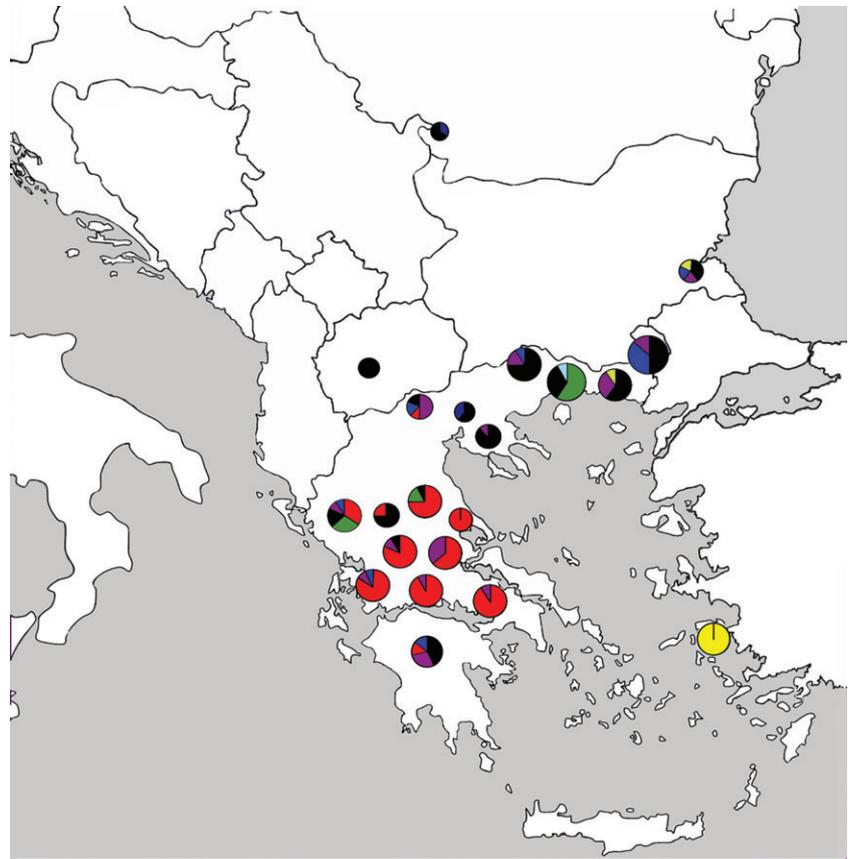
were found in wild individuals), five clustered within the E2 clade, while 12 and 126 haplotypes belonged to the Near Eastern and Asian clades, respectively. Sixty-eight haplotypes were found in Balkan wild boar, and 15 were found within the domestic pigs. Overall, 62 haplotypes (out of 205 samples analysed) were found to be unique for Balkan wild boar and only six haplotypes were shared between the Balkan samples and other European wild boar and domestic pig populations. The number of unique haplotypes found in the rest of Europe was only 22 (out of 196 GenBank wild samples from 14 countries). Furthermore, Balkan samples had a haplotype diversity ( $h$ ) similar to that of the rest of Europe ( $h = 0.892$  and  $h = 0.947$ , respectively; Table 1). If we focus on samples from within Greece, 25 haplotypes were detected exclusively in northern Greece, 19 haplotypes in central Greece and two haplotypes on the island of Samos.

Bayesian phylogenetic analyses (using the GTR + G model) revealed that despite the addition of a large number of samples and haplotypes from the Balkans, the general shape of European wild boar phylogeography remained consistent with previous studies. The majority of the wild boar haplotypes examined clustered into two major previously described clades (Giuffra *et al.*, 2000; Larson *et al.*, 2005): the European (E1) and the Near Eastern clade, both of which were supported by a posterior probability of 100% (Fig. 2). All samples studied from continental Greece and Bulgaria (with the exception of one sample from northern Greece, which possessed an Asian haplotype) grouped into the E1 clade. The four haplotypes

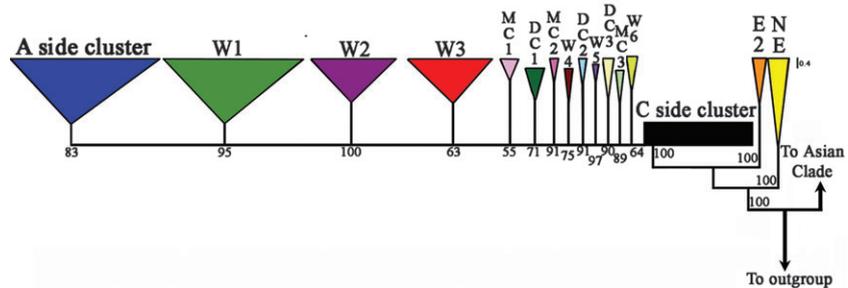
with a Near Eastern origin were observed in individuals originating from the island of Samos, which is situated close to the coast of Asia Minor.

The phylogeographical patterning of these haplotypes (Fig. 2) reveals that the E1 clade possesses numerous previously undescribed and geographically restricted subclades. Most European wild boar and domestic pig haplotypes fall into the previously described A (42 haplotypes found in 207 samples) and C (28 haplotypes found in 132 samples) clusters that are widely distributed throughout Europe (Larson *et al.*, 2005) and the Balkans, although their presence in central Greece is limited.

Apart from the A and C clusters, six clades (W1–W6) consist only of wild boar haplotypes, three clades include only domestic pig haplotypes (DC1–DC3) and three clades are mixed (MC1–MC3), consisting of both wild boar and domestic pig haplotypes (Fig. 2; for a detailed tree see Appendix S2). Of the six wild boar clades, three (W1, W2 and W3) are found exclusively in the Balkans and one of these clades (W3) is restricted to the central region of Greece, plus one extra sample in the westernmost northern population (Fig. 2). The W4 clade consists only of wild boar from Spain, W5 includes samples from Spain, Portugal and France (Larson *et al.*, 2005, 2007; Fang & Andersson, 2006; Fang *et al.*, 2006; Alves *et al.*, 2010) and W6 includes samples from the Balkans and central Europe. Regarding the mixed clades, only MC3 includes wild boar from the Balkans (as well as domestic pigs from Spain). Clades MC1 and MC2 include wild boar and domestic pigs from Spain.



**Figure 2** Map of the Balkans and a phylogenetic tree depicting the relationships between different wild boar and domestic pig clades. Phylogenetic clades W1–W6 consist of wild boar haplotypes, DC1–DC3 consist of domestic pig haplotypes, and MC1–MC3 consist of both wild boar and domestic pig haplotypes. Coloured circles within each Balkan region indicate the presence of at least one sample belonging to the coloured clades of the tree. Circle sizes are proportional to the number of samples examined from each area. The numbers below the clades represent posterior probability support values.



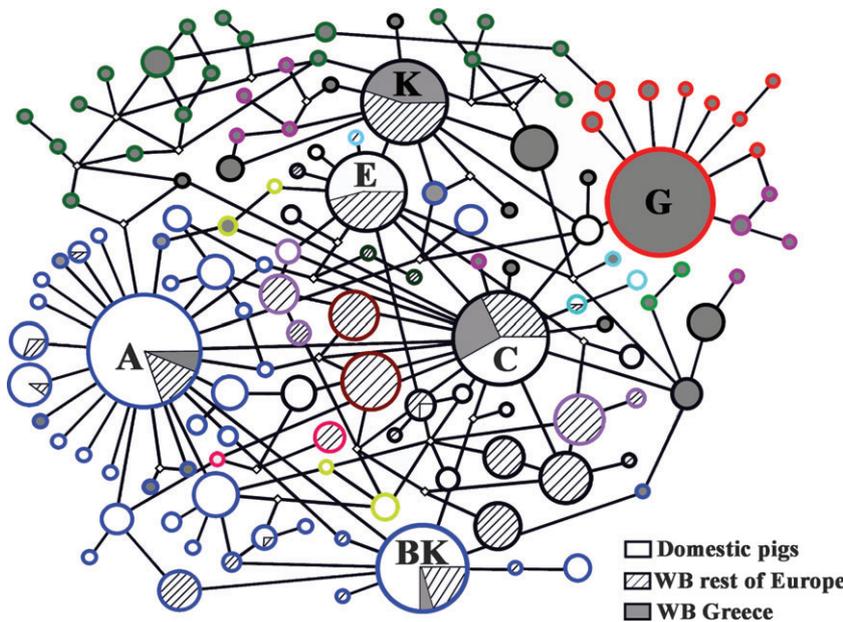
In addition to the phylogenetic tree, a network analysis (Fig. 3) provided further resolution regarding the relationships between the European and Balkan haplotypes. The pattern revealed in the network corroborates the Bayesian analyses. Six core (i.e. most frequent) haplotypes were found: haplotypes A (found in 72 individuals), G (in 63 individuals), C (in 50 individuals), BK (in 43 individuals), E (in 35 individuals) and K (in 38 individuals). Haplotypes A, C, BK and E have been previously described by Larson *et al.* (2005) to be present in European wild boar and domestic pigs. Haplotype K has also been found in previous studies (Larson *et al.*, 2005; Alves *et al.*, 2010) but here we found it in high frequency in the Balkan samples. Haplotype G (within the W3 subclade) is newly discovered, and is the most common Greek haplotype and indigenous in central Greece. In the network, this haplotype is surrounded by a starlike pattern consistent with a recent population expansion. However, despite the negative value of Tajima's *D* ( $D = -2.203$ ,  $P = 0.00$ ), a mismatch analysis

demonstrated that the central Greek population could not be fitted to the population expansion scenario ( $P = 0.000$ ) (Fig. 4a). Negative *D* values therefore indicate an excess of low-frequency polymorphisms and not a demographic expansion. On the other hand, a population growth/expansion scenario is supported for the northern Greece–Bulgaria populations by mismatch analysis ( $P = 0.711$ ) and a negative Tajima's *D* value ( $D = -1.322$ ), which marginally misses the threshold for statistical significance ( $P = 0.078$ ) (Fig. 4b).

## DISCUSSION

### The Balkans: a biodiversity hotspot for wild boar and other taxa

The southern European peninsulas – including Iberia, Italy and the Balkans – along with the Caspian/Caucasus region, acted as refugia during the LGM and many species retained regional



**Figure 3** Median-joining network in which node sizes are proportional to wild boar (WB) and domestic pig haplotype frequencies depicting the relationships between different European haplotypes in a 637-bp alignment. Coloured circles correspond to the coloured clades of the phylogenetic tree (Fig. 2). Small white dots represent inferred haplotypes.

taxonomic and genetic diversity as a result (Taberlet *et al.*, 1998; Hewitt, 1999, 2004). Our study on wild boar corroborates this general model.

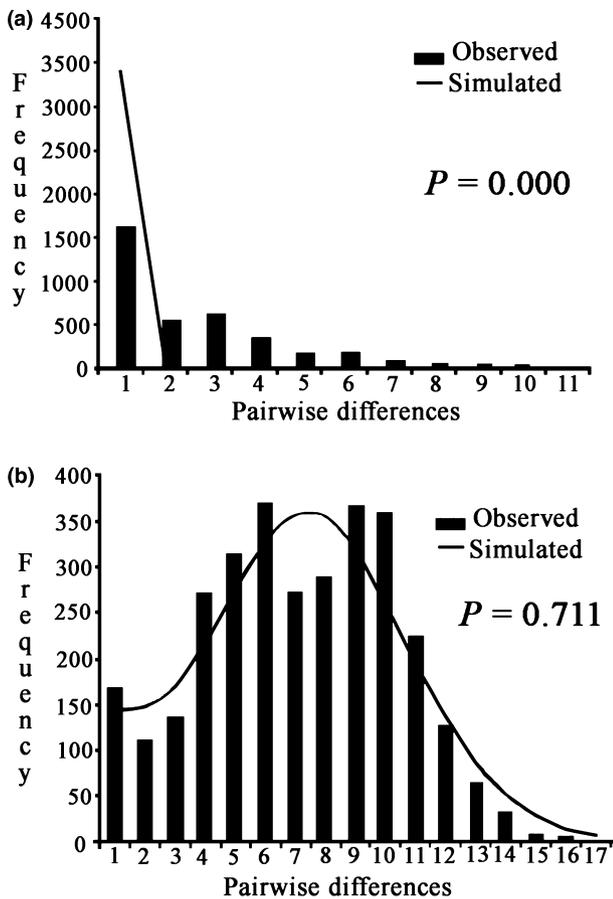
Bayesian phylogenetic and network analyses revealed that all wild boar populations from the continental Balkan region belong to the previously described (Giuffra *et al.*, 2000) E1 clade of European wild boar and domestic pigs. Despite the fact that modern endemic Italian E2 haplotypes were identified in 11,000-year-old ancient samples in present-day Croatia (Larson *et al.*, 2007) they were not found among modern Balkan samples. Instead, three new Balkan clades (W1, W2 and W3; Fig. 2) within the E1 group were recognized and clade W3 is found exclusively in central Greece (Fig. 2). Although the Balkans (and specifically Greece) occupy a significantly smaller geographical area than the rest of Europe, levels of genetic diversity within the studied wild boar population (0.892) are similar to those found in the rest of Europe (0.947), and the number of unique haplotypes (62) is larger than in the rest of Europe (22).

The existence of a large number of unique haplotypes in the Balkan refugium suggests that a large fraction of the pre-glacial wild boar diversity was maintained in this area during at least the latest glacial period. Similar cases of high biodiversity in the Balkans region have been found in numerous other species, including the brown hare (Kasapidis *et al.*, 2005; Stamatis *et al.*, 2009), yellow-necked field mouse (Michaux *et al.*, 2004), nose-horned viper (Ursenbacher *et al.*, 2008), green and fire-bellied toads (Stöck *et al.*, 2006; Hofman *et al.*, 2007), alpine newt (Sotiropoulos *et al.*, 2007), meadow grasshopper (Hewitt, 1999, 2000, 2004), marbled white butterfly (Habel *et al.*, 2005; Schmitt *et al.*, 2006) and clouded Apollo (Gratton *et al.*, 2008). These data collectively show that the Balkan Peninsula was a major European refugium during the LGM, and subsequently an important source of the post-glacial colonization of

northern Europe. High biodiversity within the Balkans can be explained by the long-term persistence of favourable environmental conditions within the refugium that allowed for the maintenance of stable and genetically variable populations (e.g. Canestrelli *et al.*, 2010).

The presence of distinct wild boar haplotypes in central Greece suggests that this area harbours a large diverse population that was not involved in the northward expansion. The pattern of northern European populations originating from the Balkans alongside the existence of unique and geographically constrained Greek populations has also been identified in other species, including the brown hare, yellow-necked field mouse, nose-horned viper, green-bellied toad, marble white butterfly and meadow grasshopper. In order to explain these patterns, a model of 'multiple refugia within refugium' has been repeatedly suggested not only for the Balkans but for other European refugia, including Iberia (Szymura *et al.*, 2000; Seddon *et al.*, 2001, 2002; Paulo *et al.*, 2002; Petit *et al.*, 2003; Sotiropoulos *et al.*, 2007; Ursenbacher *et al.*, 2008; Centeno-Cuadros *et al.*, 2009). The existence of habitat heterogeneity stimulates and maintains genetic differentiation among populations, resulting in a 'hotspot' of endemism (Blondel & Aronson, 1999; Griffiths *et al.*, 2004). The same hypothesis can also be applied to the pattern of wild boar diversity. The woodland patches distributed in the Balkans during the LGM (Sommer & Nadachowski, 2006) provided the context in which numerous haplotypes evolved.

The unique central Greek wild boar haplotypes are, however, not found in modern-day northern Europe. This can be explained by the post-glacial 'leading edge' expansion scenario proposed by Hewitt (1999) for meadow grasshoppers. According to this hypothesis, most of Europe was populated from a northern Balkan refugium, while central Greek populations were blocked from expanding by their more northerly



**Figure 4** Mismatch distribution of the mitochondrial control region in two of the studied wild boar populations: (a) central Greece and (b) northern Greece–Bulgaria. The *P*-value is used to test the hypothesis that the observed data fit the sudden expansion model.

neighbours. Nichols & Hewitt (1994) argued that post-glacial population shifts in southern Europe exhibit the ‘pioneer’ form of expansion, where populations from the northern limits of the refugial range expand rapidly before other dispersants arrive. The specific migration patterns depend upon the sharpness of the climatic changes, the topography and the dispersal and reproductive capacities of the organisms. If we assume that the wild boar colonization of Europe started from several southern refugia that hosted numerous genetically distinct populations, when the spread is rapid, one of the refugial populations may expand over the continent while the others remain in the south; once an area has been colonized, it is difficult for migrants behind the leading edge to contribute to the newly emerging population (Hewitt, 1993). As a result, populations that are behind the northern ‘front line’, particularly in the centre and southern portions of the refugium, will not be able to advance easily.

This expansion pattern has also been proposed for the Balkan populations of other species such as the marbled white butterfly and European brown hare. Schmitt *et al.* (2006) suggested that the expansion edge for the marbled white

butterfly was located in the northern part of the Balkans, while the remaining north-western Greek groups were responsible for the establishment of a differentiated Carpathian Basin population. The brown hare example possesses even more similarities to wild boar. Kasapidis *et al.* (2005) and Stamatis *et al.* (2009) identified a phylogenetically distinct group consisting of haplotypes from central Greece. The higher degree of differentiation of hares from central Greece was interpreted as a result of post-glacial movements of a north Balkan European brown hare population, which prevented the expansion of these distinct Greek groups to central Europe.

Another question regarding the expansion of wild boar in Europe concerns the contribution of each refugium. The simplest hypothesis for the wild boar recolonization of Europe is an equal contribution of Iberian and Balkan populations: Iberian boar colonized western Europe, while Balkan boar expanded into central Europe. Western European regions do appear to share haplotypes with the Iberian Peninsula (Alves *et al.*, 2010), while central European populations seem to be closer to the Balkans. This scenario is supported by the fact that only C-like haplotypes are found in Iberian wild boar and domestic pigs, while both C- and A-like haplotypes were detected in the Balkans and central Europe (Figs 2 & 3). A possible explanation for this pattern begins with the existence of C-like haplotypes throughout Europe before the LGM, consistent with their basal placement in the phylogenetic tree. Climatic oscillations during the Pleistocene resulted in the contraction of wild boar populations to different refugia; A-like haplotypes appeared in the Balkans and co-existed with the C-like group during the glaciations. As the climate warmed, Balkan and Iberian populations spread northwards colonizing central Europe but A-like haplotypes were prevented from entering the western European regions because of the already established C-like haplotypes. A similar hypothesis has been proposed by Alves *et al.* (2010), suggesting that the Iberian populations could have contributed to the post-glacial colonization by spreading C-like haplotypes. Sommer & Nadachowski (2006) identified another wild boar refugium in southern France (Dordogne region) that could also have contributed to the spreading of C-like haplotypes.

Dating the demographic events that shaped the genetic diversity of wild boar has proved controversial. Previous studies on wild boar and domestic pigs (Kim *et al.*, 2002; Fang & Andersson, 2006; Scandura *et al.*, 2008) have employed molecular clock analyses using imported evolutionary rates. Given that observed rates of evolution increase as population divergences approach the present, Ho & Larson (2006) demonstrated that molecular clocks may not be valid when dating recent events (i.e. < 2 million years). In addition the coalescence of lineages need not coincide with population splits (Taberlet *et al.*, 1998). In this respect, the use of reliable ancient DNA and radiocarbon dated sequences will be very useful in producing accurate date estimates for the demographic scenario described above.

## Absence of introgression of wild boar Near Eastern haplotypes in Europe

In addition to the Balkans, Anatolia and the Near East have also acted as refugia (Hewitt, 1999; Randi, 2007; Schmitt, 2007) and these regions contain a high proportion of unique haplotypes of many species, including European hedgehogs (*Erinaceus* spp.) (Santucci *et al.*, 1998; Hewitt, 1999, 2000, 2004; Seddon *et al.*, 2001; Berggren *et al.*, 2005) and brown bears (*Ursus arctos*) (Taberlet *et al.*, 1998; Hewitt, 1999, 2000; Korsten *et al.*, 2009). Wild boar sequences from the island of Samos revealed the existence of Near Eastern mtDNA haplotypes similar to those found in modern wild boar from Turkey and Iran (Larson *et al.*, 2007), although this is not surprising given the geographical proximity of this island to the Anatolian Peninsula. In other species, such as the newt *Triturus* sp. (Taberlet *et al.*, 1998) and the meadow grasshopper *Chorthippus parallelus* (Hewitt, 1999), the Turkish and Balkan populations seem to be closely related, suggesting recent separation or gene flow between them during the last glaciation, when the Marmara Sea had receded creating a land bridge between the two regions (Tzedakis *et al.*, 1997; Aksu *et al.*, 1999).

Hybrid zones have also been recognized for other species (e.g. yellow-necked field mouse, European brown hare) near the Bosphorus including Thrace and eastern Bulgaria (Michaux *et al.*, 2004; Kasapidis *et al.*, 2005; Stamatis *et al.*, 2009). No study of modern wild boar has revealed Near Eastern haplotypes in Europe, and despite our extensive sampling in the Balkans, our study is no different. It is possible that the presence of established wild boar in the Balkans prevented the colonization by new invaders and that even if some migration from Asia Minor over the land bridge occurred there has been no lasting effect on the indigenous maternal DNA.

## Impact of domestication processes on present-day wild boar genetic structure

Only a small proportion of the wild individuals that we analysed show the effects of domestic pig introgression. None of the southern or central Greek wild boar share haplotypes with modern domestic pigs. Additionally, those northern Greek wild boar that do share haplotypes with domestic pigs possess the haplotypes that are the most common in Europe, suggesting a recent hybridization with European domestic pigs. This is supported by the presence of an Asian haplotype in one individual which almost certainly derived from hybridization with an improved European breed with a history of Asian maternal ancestry. Our results agree with those of Scandura *et al.* (2008, 2011a), who suggest that the impact of more recent human activities on the genetic structure of wild boar is limited.

## CONCLUSIONS

The Balkan Peninsula was clearly an important ice age refugium and the home of the source populations of many

species that colonized Europe following the LGM (Hewitt, 1999, 2000). The presence of numerous unique wild boar haplotypes in the Balkans supports this model and the pattern of its genetic variation is probably the result of a leading edge expansion. Although this study establishes the general picture of refugial populations of wild boar in the Balkans, a wider sampling effort that includes central European populations is necessary for post-glacial routes to be clarified. Unlike some other species, indigenous Anatolian wild boar populations did not contribute to the modern maternal genetic diversity of Balkan or European wild boar. They may have contributed nuclear DNA, however, and given the large sex dispersal differences (Keuling *et al.*, 2010) and hybridization with domestic pigs, the use of nuclear (e.g. microsatellite or single nucleotide polymorphism) markers (e.g. Vernesi *et al.*, 2003; Scandura *et al.*, 2008, 2011b; Ferreira *et al.*, 2009; Hajji & Zachos, 2011) will help to detect this possible contribution and the presence of hybrid zones between the Balkan and Asia Minor populations.

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## SUPPORTING INFORMATION

Additional Supporting information may be found in the online version of the article:

**Appendix S1** Analytical list of wild boar and domestic pig mitochondrial control region sequences obtained in this study or downloaded from GenBank.

**Appendix S2** A detailed version of the phylogenetic tree shown in Fig. 2 of the main text, containing 245 haplotypes found in European wild boar and domestic pigs.

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## BIOSKETCH

**Panoraia Alexandri** is a PhD student studying the genetic structure of Greek wild boar. This is well integrated in the focus of the research group from the School of Biology of the Aristotle University of Thessaloniki (Greece), i.e. the genetic analysis of the diversity of Greek animal species.

Author contributions: P.A., A.T., E.C., N.P. and C.T. conceived the study; P.A., E.C., P.P. and C.T. collected the specimens; P.A. conducted the DNA analyses; P.A., A.T. and S.P. analysed the data; and P.A., A.T., S.P., G.L., T.J.A. and C.T. wrote the paper. All authors read, commented on and approved the final manuscript.

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