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ORIGINAL RESEARCH ARTICLE

Genetic structure of *Apis mellifera macedonica* in the Balkan Peninsula based on microsatellite DNA polymorphism

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Summary

The genetic variability of honey bees (*Apis mellifera* L.) from south eastern Europe was investigated using microsatellite analyses of 107 samples from Albania, the Republic of Macedonia, Greece and Bulgaria together with 42 reference samples (*Apis mellifera carnica*) from Slovenia. Genetic structure and spatial analyses of the microsatellite data showed a clear distinction between the Slovenian bees and all other populations, and confirmed the existence of *Apis mellifera macedonica* as an indigenous honey bee population in the regions that were sampled. In most areas however, varying degrees of introgression with *A. m. carnica* alleles could be observed, probably as a consequence of propagating imported queens. Within *A. m. macedonica*, a certain degree of subdivision between the honey bee populations from Bulgaria and the other regions was detected, confirming earlier reports of variation within this subspecies.

Estructura genética de *Apis mellifera macedonica* en la península de los Balcanes basada en el polimorfismo de ADN microsatélite

Resumen

La variabilidad genética de las abejas melíferas (*Apis mellifera* L.) de Europa sudoriental se investigó mediante el análisis de microsatélites de 107 muestras procedentes de Albania, la República de Macedonia, Grecia y Bulgaria, junto con 42 muestras de referencia (*Apis mellifera carnica*) de Eslovenia. La estructura genética y el análisis espacial de los datos de microsatélites mostraron una clara distinción entre las abejas eslovenas y las demás poblaciones, y confirmaron la existencia de *Apis mellifera macedonica* como una población de abejas de la miel indígena en las regiones que se muestrearon. En la mayoría de las áreas, sin embargo, se pudieron observar diferentes grados de introgresión de alelos de *A. m. carnica*, probablemente como consecuencia de la propagación de reinas importadas. Dentro de *A. m. macedonica*, se detectó un cierto grado de subdivisión entre las poblaciones de abejas melíferas de Bulgaria y de las otras regiones, lo que confirma informes anteriores de la variación dentro de esta subespecie.

Keywords: *Apis mellifera macedonica,* diversity, Balkans, microsatellite

Introduction

The native range of the western honey bee *Apis mellifera* covers a wide range of climatic and vegetation conditions, including all of Europe, Africa, and western and central Asia. Honey bee phylogeography has been comparatively well studied, and to date 27 subspecies, grouped into four major lineages have been recognised (Ruttner 1988; Garnery *et al.*, 1992; Whitfield *et al.*, 2006; Sheppard and Meixner, 2003; Meixner *et al.*, 2011).

After the end of the last ice age, honey bees repopulated the Balkan Peninsula from their Pleistocene refuges in southern Greece, and gave rise to the closely related subspecies *A. m. carnica, A. m. cecropia* and *A. m. macedonica* (summarised in Ruttner, 1988). Although substantial geographic variation in the honey bee populations of this region was noted early on (Adam, 1983; Ifantidis, 1979), *A. m. macedonica* was described only in 1988 as a separate subspecies, based on a comprehensive morphometric analysis (Ruttner, 1988). Its natural range has been reported to cover the south eastern part of the Balkan Peninsula, from Albania, Greece, the Republic of Macedonia to Bulgaria, but reaching as far as the Ukraine and southern Poland in the north east (Ruttner, 1988).

Despite this wide range of *A. m. macedonica,* so far only few studies, mostly focusing on a limited aspect or geographic area, have been conducted (Dedej *et al.*, 1996; Bouga *et al.*, 2005a,b; Ivanova *et al.*, 2007; Uzunov *et al.*, 2009; Stevanovic *et al.*, 2010; Ivanova *et al.*, 2012) and thus, comparatively little information is available regarding the geographic and genetic variability of *A. m. macedonica* and its range limits.

Beekeeping is a popular occupation in south eastern Europe, with the beekeeping industry mostly relying on stationary apiaries and often still using traditional beekeeping management practices. However, modern beekeeping, including migration and queen importation, is becoming increasingly common (Bouga *et al.*, 2005a,b; Uzunov *et al.*, 2009; Stevanovic *et al.*, 2010). The native bee is still widely used over the entire region, but there are concerns that it may be increasingly subjected to introgression of foreign genetic material and hybridisation with other subspecies.

In this paper, we present the results of a comprehensive microsatellite analysis of honey bee populations from Albania, Bulgaria, Greece and the Republic of Macedonia in south eastern Europe. We report new information regarding the genetic composition of the subspecies *A. m. macedonica* and its relation to the neighbouring subspecies *A. m. carnica*. We demonstrate the usefulness of R package 'adegenet' to reveal cryptic patterns of population structure within the *A. m. macedonica* subspecies.

Material and methods

Sampling

Honey bee workers were sampled in 2008 and 2009 from eastern Albania, the Republic of Macedonia, northern Greece, Bulgaria, and Slovenia. In total, 149 samples of workers were collected from 90 locations (Table 1), where each sample represented one colony. Worker bees were individually stored in absolute ethanol (96%). All samples, except for two apiaries in northern Greece, were collected from stationary bee yards using traditional beekeeping practices and a very low possibility of using imported queens. Six samples from northern Greece were collected from migratory beekeeping operations from the region of Volos, central Greece. Detailed sample information including GPS data is available online:
http://www.ibra.org.uk/downloads/20140515/download

Table 1. Number of localities and number of colonies (n) sampled from eastern Albania, Macedonia, northern Greece, Bulgaria, and Slovenia.

Country	**Locations**	***n***
eastern Albania	6	14
Macedonia	22	50
northern Greece	4	12
Bulgaria	28	31
Slovenia	30	42

DNA extraction and genotyping

DNA was extracted from one bee per colony using DNeasyTM 96 Blood & Tissue Kit (Qiagen). The flight muscles of each bee were removed from the thorax using clean forceps and introduced into DNeasy 96 tubes. DNA was extracted according to the kit instructions and it was eluted into 100 µl sterile water and stored at -20°C. Four separate PCR reactions were carried out with fluorescent-tagged primer pairs with six to nine primers multiplexed per reaction. A total of 25 loci were genotyped (Table 2) (Estoup *et al.*, 1995; Solignac *et al.*, 2003). For locus A014, the primer set was redesigned to simplify the scoring, thereby reducing the length of the amplicon from 230 bp to 117 bp. The new primers used were F: ACG CGG CGA TCG TAA AA and R: CCA CCG TGC GAT GAC G. Genotyping was carried out on a 96 capillary ABI 3730xl Sequencer (Applied Biosystems). Alleles were initially scored automatically by GeneMapper software, then verified twice by eye and corrected manually if required.

Table 2. List of microsatellite loci used in this study.

Microsatellite loci							
A8	A14	A24	A29	A79	A88	A133	Ac11
Ac88	Ac139	Ac306	Ap15	Ap85	Ap90	Ap223	Ap224
Ap226	Ap249	Ap273	Ap274	At168	At188		

Non-spatial analysis

The GenAlEx 6/6.5 program (Peakall *et al.*, 2006; Peakall *et al.*, 2012) was used with Excel™ to organise the data and to compute frequency -based statistics and AMOVA. Subsequently, the data were analysed using the STRUCTURE 2.3.3 software (Pritchard *et al.*, 2000), which implements a Bayesian method using Monte Carlo Markov Chain (MCMC) approach to cluster individuals. Based on the number of clusters defined by the user, STRUCTURE randomly allocates individuals to clusters in order to minimise the Hardy-Weinberg disequilibrium and gametic phase disequilibrium between loci within clusters. The MCMC was run for 1,000,000 iterations with a 100,000 burn-in period, with the number of clusters (K) ranging from 1-7. At least 2 repeats were performed to check the convergence of likelihood values for each K. Six repeats were carried out for each value of K from 2 to 4. The results from several runs for a given K were aligned for visualisation using the software CLUMPP (Jakobsson *et al.*, 2007). Values which maximised the likelihood of the data for each K were taken as the most probable clustering. They were visualised as proposed by Evanno *et al.* (2005). Principal Component Analysis (PCA) was carried out with cantered data using the packages adegenet (Jombart, 2008) and ade4 (Dray and Dufour, 2007) of the R software (Ihaka *et al.*, 1996). The R software was also used to plot the final STRUCTURE barplots.

Spatial analysis

Spatial analysis was carried out in R (Development Core Team, 2012) using the package adegenet (Jombart, 2008). Missing genotype values were replaced by mean values for each locus. Spatial coordinates in Longitude-Latitude were converted to Universal Transverse Mercator (UTM) coordinates for UTM zone 34. All coordinates were jittered (shifted randomly) by a factor of 10 (approximately 100 m) to avoid zero geographical distances between samples from identical coordinates. Spatial Principal Component Analysis (sPCA) was carried out using Delaunay triangulation as the connection network. The variance was plotted against spatial autocorrelation (Moran's I) to produce a screeplot which was used to visually estimate if existence of general spatial structures could be inferred. Monte Carlo tests were performed using genetic data and spatial weights for 10000 iterations to statistically test the presence of global and local spatial structure.

Background maps of political boundaries were obtained from Global Administrative Areas database (GADM v1.0, 2011) and plotted using the R package PBSmapping (Schnute, 2010). The eigenvectors for each eigenvalue scores (retained scores 1 to 5) obtained from the sPCA analysis were plotted against the geographical coordinates.

To validate the outcome of the sPCA, the individuals were separated into three clusters based on the sPCA clustering. Thus, three clusters were defined as: (a) bees labelled as white squares on Fig. 5 (n = 52), (b) bees labelled as black squares on Fig. 5 (n = 55) and (c) Slovenian bees (*carnica* bees) (n = 42). For these three clusters, an AMOVA was performed in GenAlex 6.5 with 10000 permutations.

Results

The number of alleles detected in all analysed workers varied between 1 (Ap274 in Macedonia and Greece, Ap15 in Albania) and 23 (A29 in Slovenia). The average allele number across all loci in the analysed populations was 5.08 (± 0.30) and ranged from 3.88 (± 0.55) (Albania) to 6.16 (± 0.85) (Slovenia). The overall expected heterozygosity (He) ranged from 0.415 (± 0.05) (Albania) to 0.53 (± 0.04) (Bulgaria). The allele frequencies at each locus, the number of alleles per locus, and the observed and expected heterozygosity for each population are available online: http://www.ibra.org.uk/downloads/20140515/download

The PCA plot showed the Slovenian bees (*carnica* bees) to be clearly distinct from the others (Fig. 1). No obvious clusters could be identified within non-*carnica* bees using the PCA. STRUCTURE was carried out from K = 1 to K = 7 of which K = 2 to K = 4 is shown in Fig. 1. The number of clusters detected by the method proposed by Evanno *et al.* (2005) showed the highest ΔK at K = 3. The results for K = 2 already show a clear separation between carnica and non-carnica populations (Fig 2, A). However, introgression with *A. m. carnica* alleles was detected in several bees from Albania, the Macedonia and Bulgaria. For K = 3 (Fig. 2, B) a subdivision of the non-carnica bees into two subgroups could be identified. The geographical distribution of the two non-carnica clusters is not visible from this figure. For K = 4 (Fig. 2, C), no obvious additional cluster could be observed.

The spatial analysis of the same dataset with the R package adegenet revealed a mostly concurrent population structure, with the find.clusters function estimating the number of clusters at K = 3. The screeplot (Fig. 3) showed the eigenvalue of the first global score λ1 to be clearly distinct in terms of geographical and genetic variance, and a significant global spatial structure was revealed by the global Monte-Carlo test structure ($p < 0.001$). Mantel test showed a highly significant (P = 0.0002) correlation of genetic distance with geographic distance. The Mantel test was not significant when comparing only non-carnica bees (P = 0.07).

In Fig. 4, the sPCA global score 1 was plotted against spatial coordinates as black and white squares differentiating two populations in a single image. Positive values are shown as black squares while negative values are shown as white squares with black outlines. The size of the square denotes the probability with which the given sample falls into the indicated cluster. As expected from the screeplot, a distinct geographical separation between carnica and non-carnica populations is evident. This is similar to the STRUCTURE result for K = 2 (Fig. 2, A).

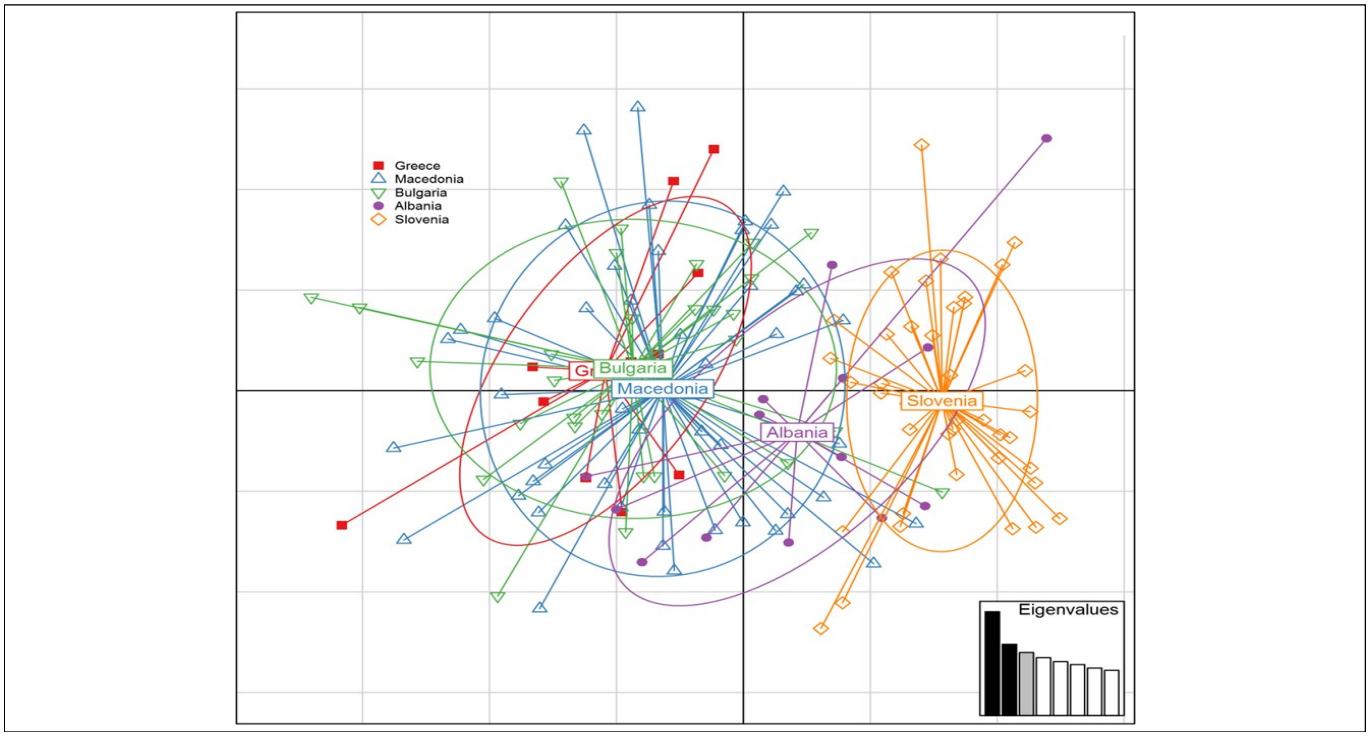

Fig. 1. PCA plot of the 149 bees from five locations (Greece (n = 12), Macedonia (n = 50), Bulgaria (n = 31), Albania (n = 14), Slovenia (n = 42)). Principal component axis 1 and axis 2 are shown here. The Slovenian bees (*carnica* bees) are easily identifiable as a separate cluster. Sub-structures within non-*carnica* bees are not easily recognisable.

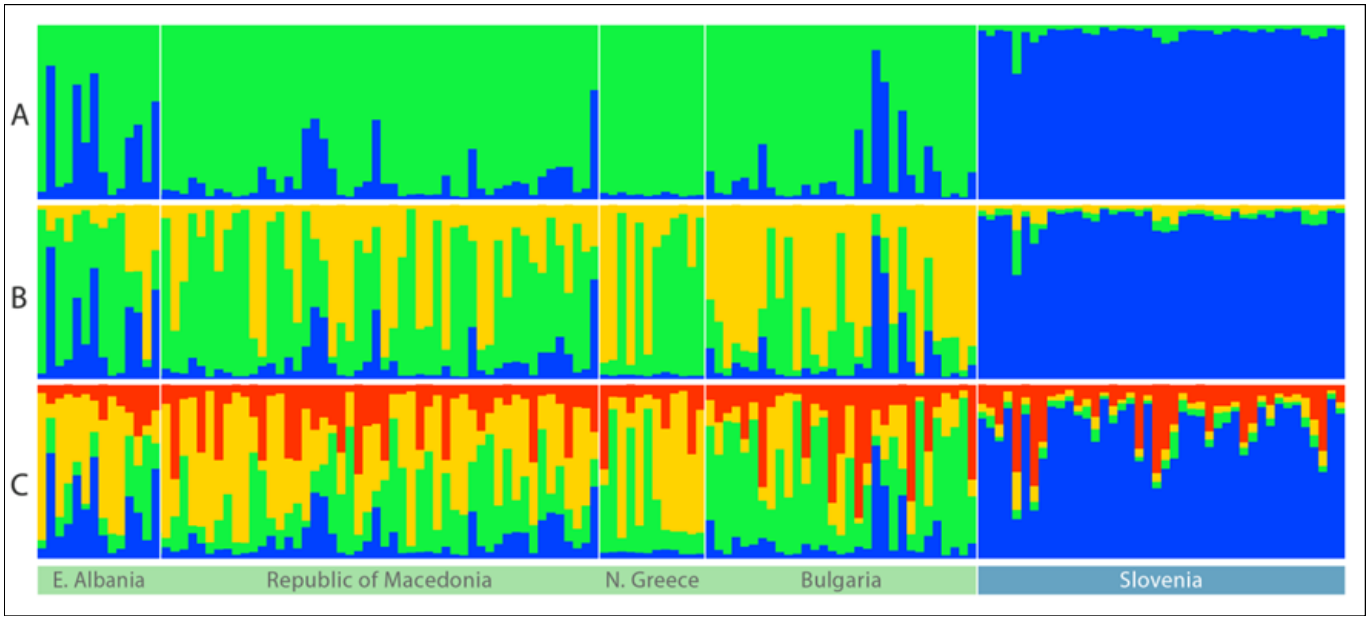

Fig. 2. STRUCTURE barplots for *K* = 2 to *K* = 4 (top to bottom). The origins of the bees are labelled below separated by thin white vertical lines. Plot colours are as follows: Blue; cluster 1, green; cluster 2, yellow; cluster 3, red; cluster 4. Labelling colour blue denotes *carnica* bees while green denotes non-*carnica* bees. (A) shows K = 2, where the Slovenian bees (*carnica* bees; cluster 1) readily show a very distinct and separate cluster. (B) shows K = 3, where the non-carnica bees split up into the new cluster 3. The Bulgarian bees show a higher percentage of allocation to cluster 3 than other non-*carnica* bees. This may indicate a sub-structure within the non-*carnica* bees. (C) Cluster 4 does not show any obviously identifiable pattern.

For the second sPCA global score (λ2), we observed distinct clustering within the non-carnica bees (Fig. 5). One of these clusters was mostly confined to the western part of our sampling, while the second cluster was localised in the central and eastern parts of Bulgaria. The population structure of *A. m. macedonica* emerging from this analysis at the individual level was similar to that determined from STRUCTURE with K = 3 (Fig. 2, B). The AMOVA showed significant ($P < 0.01$) separation of the three clusters with FST values of (a to b) 0.032, (b to c) 0.079 and (a to c) 0.037.

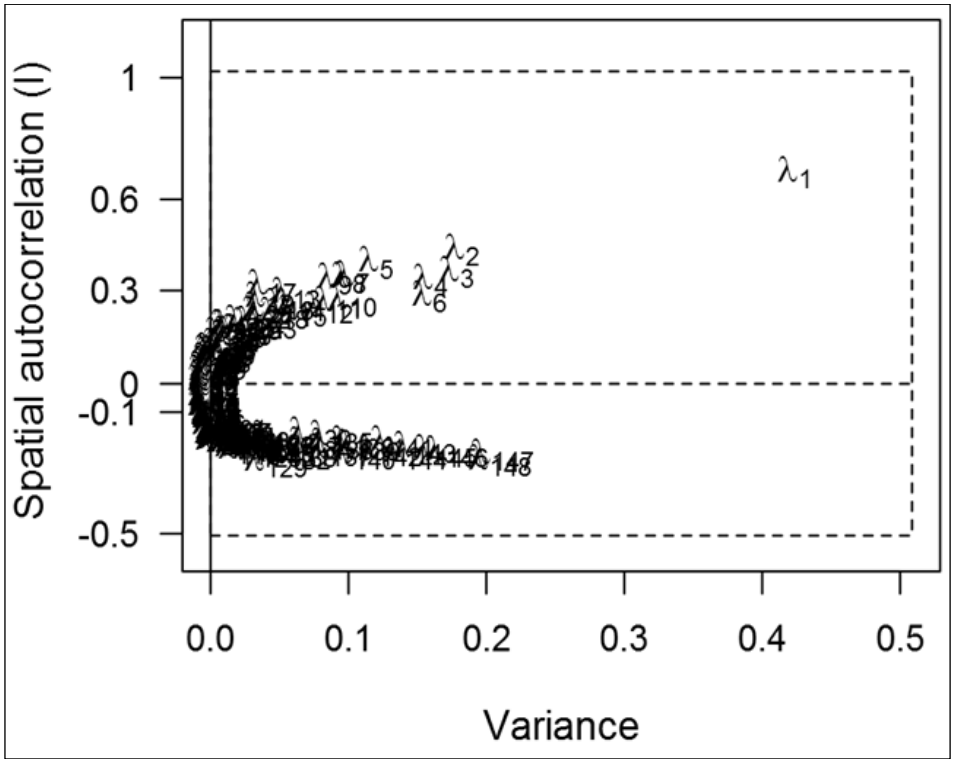

Fig. 3. Screeplot for the total dataset showing sPCA eigenvalues with variance on the x-axis and spatial autocorrelation (Moran's I) on the y-axis. The positive scores on the y-axis are referred to as global scores while the negative scores are referred to as local scores. The first global score (λ_1) having the highest value for variance and spatial autocorrelation can be easily distinguished from the other scores. Hence, λ_1 may be interpreted as a distinct population structure. The local scores do not show any obvious feature.

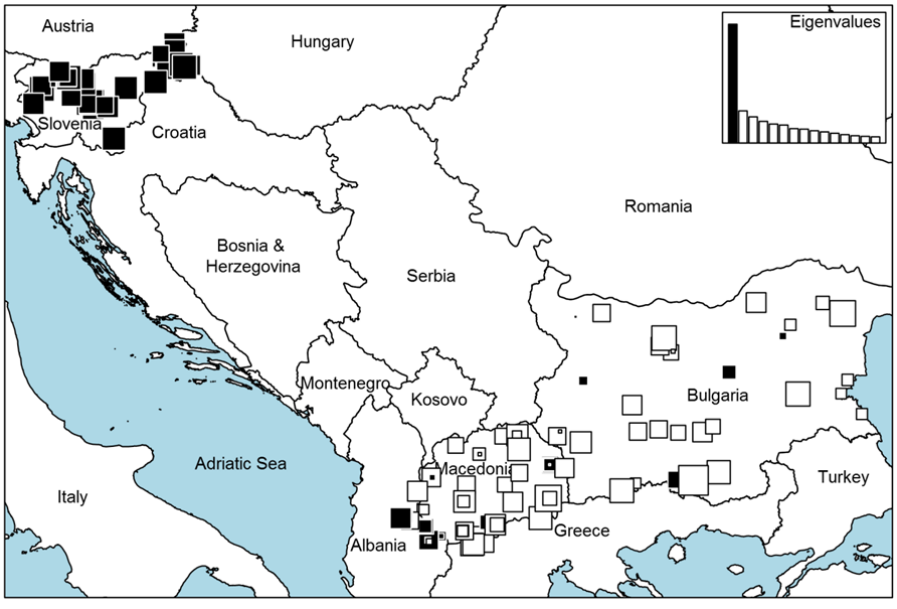

Fig. 4. sPCA global score 1 plotted out to spatial coordinates. The black and white squares represent two distinct clusters based on genetic and as well and spatial data. The size of the squares denote the probability with which an individual belongs to that cluster. Here, the Slovenian bees (*carnica* bees) are clearly different from non-carnica bees in the South. Inset top right shows the first 15 sPCA positive (global) scores.

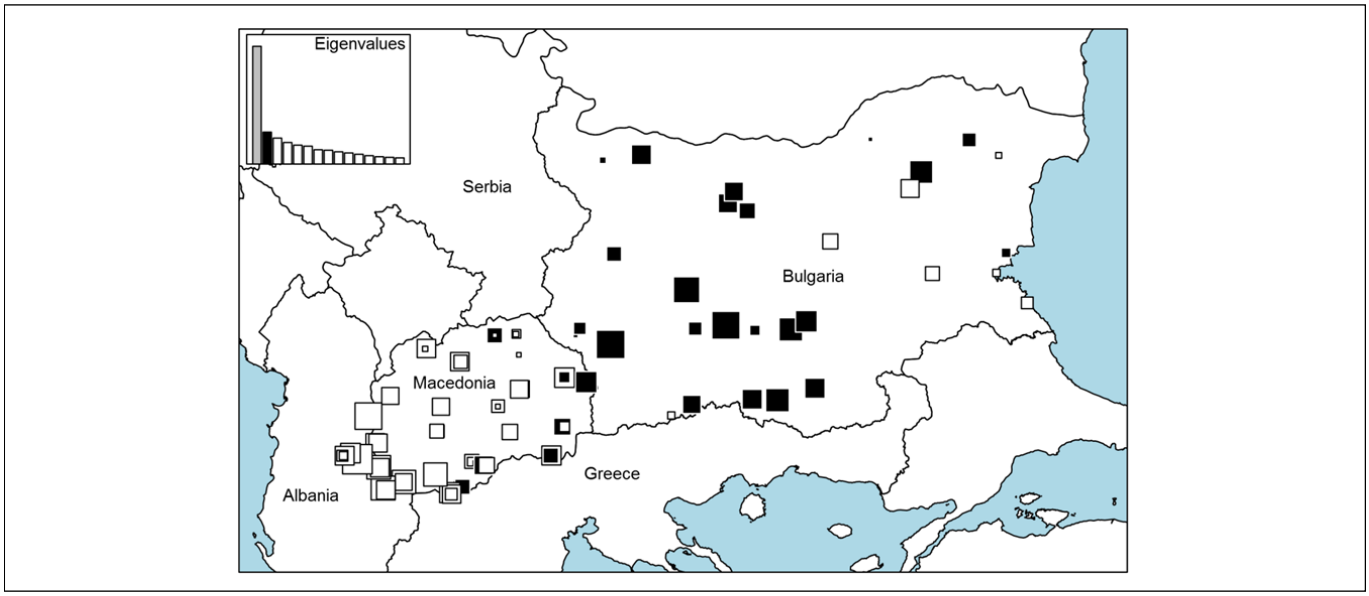

Fig. 5. sPCA global score 2 plotted out to spatial coordinates showing separation of non-carnica bees into two sub-clusters. Inset top left shows the first 15 sPCA positive (global) scores.

Discussion

This study presents the first comprehensive genetic analysis of the indigenous population of *A. m. macedonica*. Based on 25 microsatellite loci, we analysed the population structure over most of its native range, including some previously unstudied areas.

Our microsatellite results show that the honey bees of our study region, corresponding to the subspecies *A. m. macedonica* as described by Ruttner (1988) based on morphometric analysis, are genetically clearly distinct from *A. m. carnica*. Thus, they confirm the existence of two distinctive subspecies, *A. m. carnica* and *A. m. macedonica* in the Balkan Peninsula, with *A. m. macedonica* appearing as a contiguous population in the entire region we studied. However, it is also evident that populations from different regions show varying degrees of introgression from *A. m. carnica*, as has been reported before (Dedej *et al.*, 1996; Uzunov *et al.*, 2009; Stevanovic *et al.*, 2010; Munoz *et al.*, 2012). The Carniolan influence to the honey bee population in the Republic of Macedonia can be partly explained as consequence of long-term propagation of imported *A. m. carnica* queens. The situation in Albania is more difficult to explain, because hardly any information on past and present breeding practices and importation of queens is available.

Upon closer analysis, with Structure K = 3 and the second global score of the spatial analysis, a clear subdivision within *A. m. macedonica* became visible. Here, the bees from Bulgaria appeared separate from the bees collected in the other regions; however, compared to the clear contrast between *A. m. carnica* and *A. m. macedonica* the differentiation is far less pronounced. This result is in agreement with previously published data based on allozymes (Ivanova, 2010; Ivanova *et al.*, 2010, 2012) and variation of mitochondrial DNA (Martimianakis *et al.*, 2011), reporting different genetic characteristics in *A. m. macedonica* from Bulgaria compared to other *A. m. macedonica* and *A. m. carnica*. Although Ruttner (1988), in his morphometric analysis and description of *A. m. macedonica*, gave no indication of geographical variation within the subspecies, Petrov (1996) noted specific variation of morphometric characteristics in Bulgaria and suggested to consider the bees of Bulgaria as separate subspecies "*A. m. rodopica*". While our results based on microsatellite analyses indeed show a certain degree of differentiation between the bees of Bulgaria and *A. m. macedonica* from other regions, this does not necessarily translate into a confirmation of Petrov's hypothesis of a separate subspecies in Bulgaria. To verify this hypothesis, detailed analyses of the honey bee populations of Bulgaria with all neighbouring geographical regions will be needed.

Previous results based on morphometric analyses suggested that *A. m. macedonica* also occurs far to the north of the region we studied, reaching as far as the Ukraine where it forms a broad zone of hybridisation with *A. m. mellifera* (Meixner *et al.*, 2007). Based on mtDNA evidence, Bouga *et al.* (2005b) demonstrated the existence of a sharp line of differentiation to the south, between bees from northern Greece, where *A. m. macedonica* is considered native, and samples from central Greece and the islands in the Aegean Sea. According to Ruttner (1988), *A. m. macedonica* is also found in the Thrace region towards the east where it meets with *A. m. anatoliaca*. In contrast to previous publications, where the line of hybridisation between *A. m. carnica* and *A. m. macedonica* was hypothesised to run through the Republic of Macedonia and Albania (Ruttner, 1988; Dedej *et al.*, 1996; Uzunov *et al.*, 2009), our results demonstrated that the entire bee population of Macedonia and probably a substantial part of Albania has to be regarded as *A. m. macedonica.* This result is also supported by the conclusions of Stevanovic *et al.* (2010) and Munoz *et al.* (2012) who reported *A. m. macedonica*, and not *A. m. carnica,* as the native bee of the south part of Serbia.

In this study we have shown that sub-structures within non-*carnica* bees could not be easily identified using tools such as PCA and STRUCTURE. These more cryptic patterns were revealed using sPCA, which demonstrates the value of 'adegenet' in understanding complex population structures. Our results provided the first contribution based on microsatellite variation to the understanding of the genetic structure of *A. m. macedonica* in its native area of distribution, revealing that its genetic composition is obviously distinctive from the neighbouring *A. m. carnica*. With the demonstration of an evident genetic substructure within *A. m. macedonica* our results also highlight the need for additional comprehensive studies in the region, preferably combining molecular, morphological and ethological approaches. The need for protecting and conserving the genetic and geographic variability of *A. m. macedonica* emerged as an ultimate and irrevocable goal for public, professional and scientific communities in the countries involved.

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