Journal of Biogeography (J. Biogeogr.) (2014)

ORIGINAL ARTICLE

The contribution of Anatolia to European phylogeography: the centre of origin of the meadow grasshopper, *Chorthippus parallelus*

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ABSTRACT

Aim *Chorthippus parallelus* is one of the classic model systems for studying genetic structure and phylogeography in the Western Palaearctic. Here, we investigate the regional genetic differentiation of *C. parallelus* and evaluate the historical and evolutionary processes responsible for such genetic structuring, test the nature of the Turkish Straits system as a barrier to dispersal, and explore the contribution of Anatolian populations to the biodiversity of the Western Palaearctic.

Location Western Palaearctic.

Methods We incorporated sequence data from dense sampling of the phylogeographically important Anatolian region with both previous and newly obtained data of the nuclear fragment *cpnl-1* and the mitochondrial fragment *COI–tRNALeu–COII*. In total, 1049 sequences of *cpnl-1* from 33 regions were analysed to investigate the genetic diversity, genetic structuring and phylogeography of *C. parallelus* across its distributional range. The mtDNA region was additionally used to test whether the Turkish Straits system acts as a barrier.

Results The analyses revealed that not all southern refugial populations of *C. parallelus* have contributed equally to the post-glacial recolonization of Europe. Four genetic clusters across the species' range were recovered: cluster A (eastern part of the Anatolian Diagonal); cluster B (western part of the Anatolian Diagonal); cluster C (Spain, Italy, southern Balkans, west part of Anatolia and Russia); and cluster D (covering the entire distributional range of the species). The Turkish Straits system has been a weak barrier to dispersal by *C. parallelus*, allowing gene flow from Anatolia to the Balkans.

Main conclusions The current patterns of genetic structuring of *C. parallelus* were best explained by multiple expansion and contraction events. Anatolia has been well connected to the Balkans, contributing genetically to the establishment of central and northern European populations prior to the Holocene. The Anatolian refugium is suggested to be the centre of origin for Western Palaearctic *C. parallelus* diversity rather than a Balkan refugium.

Keywords
Anatolia, *Chorthippus parallelus*, centre of origin, genetic structuring, glacial refugia, meadow grasshopper, phylogeography, Turkish Straits system, Western Palaearctic.

INTRODUCTION

Phylogeographical studies of Western Palaearctic lineages have allowed several important generalizations concerning the history of temperate species (Hewitt, 1996, 2000; Taberlet *et al.*, 1998). One such generalization is that the Pleistocene glacial cycles had significant effects on the present distribution of many species, after the biodiversity of northern areas

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doi:10.1111/jbi.12332

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was largely removed during glacial periods and re-established by founder populations from southern refugia during the warmer interglacials (Hewitt, 1996, 1999, 2000; Taberlet *et al.*, 1998; Schmitt, 2007). Iberia, Italy, the Balkans and North Africa have been identified as the likely glacial refugia of the Western Palaearctic (Hewitt, 1996; Atkinson *et al.*, 2007; Médail & Diadema, 2009; Çıplak *et al.*, 2010; Husemann *et al.*, 2014). Three common routes of recolonization from southern refugia to northern areas have been recognized (Hewitt, 1999), although other routes may also have played a role (Taberlet *et al.*, 1998; Rokas *et al.*, 2003; Schmitt, 2007). Although not rigorously tested, the hypothesis that Anatolia was an important glacial refugium has also been suggested (Hewitt, 1996; Rokas *et al.*, 2003; Çıplak, 2004, 2008; Korkmaz *et al.*, 2010; Ansell *et al.*, 2011). The geographical location of Anatolia, together with its complex palaeogeographical history, heterogeneous topography and climate, contributes to the high levels of endemism and global importance of its biodiversity (Şekercioğlu *et al.*, 2011). Studies focusing on several lineages indicate that Anatolia harbours a high level of genetic diversity (Rokas *et al.*, 2003; Gündüz *et al.*, 2007; Dubey *et al.*, 2007; Fritz *et al.*, 2009; Stamatis *et al.*, 2009; Akın *et al.*, 2010; Çıplak *et al.*, 2010; Mutun, 2011), but in general the region has been underrepresented in regional phylogeographical studies. With the effects of the climatic shifts of the Pliocene and Pleistocene, vertical range changes may be expected because of the heterogeneous topography. During the warm interglacials in particular, populations that prefer cold conditions would have been isolated and may have diverged on top of high mountains or 'sky islands', and this feature may suggest a complex refugial system for Anatolia (Çıplak, 2004, 2008).

The meadow grasshopper, *Chorthippus parallelus* (Zetterstedt, 1821), has been a key example of phylogeographical structuring and gives its name to one of Hewitt's three refugial expansion models (Hewitt, 1999). Phylogeographical studies of *C. parallelus* have used both mitochondrial DNA and nuclear loci, and suggest a division into three major genomic units – Iberia, Calabria (southern Italy), and the populations in the rest of the species' range (Cooper *et al.*, 1995; Lunt *et al.*, 1998). Besides the acceptance that the present European ranges of species have originated by postglacial expansion from the Balkans, Hewitt (1996) also suggested that Anatolian populations may have survived the glacial cycles and contributed to the repopulation of Europe. Some Anatolian specimens were included by Cooper *et al.* (1995), but this sampling was insufficient to properly represent either the genetic diversity or the geographical fragmentation of the species within Anatolia (Korkmaz *et al.*, 2010). The idea that the species may have originated in Anatolia has therefore remained largely untested. An investigation into the relationships among Anatolian populations may also contribute to our understanding of the source of the diversity of *C. parallelus* during periods of expansion.

The other issue to be considered is the direction of range change during either cold or warm periods. It is generally accepted that during interglacial periods the northern edge of the population was the leading or expanding edge and the southern edge was the eroding or rear edge, with the opposite in cold periods (Çıplak, 2004, 2008; Hampe & Petit, 2005). Some assumptions of the rear edge/leading edge hypothesis, however, appear to be in conflict with the characteristics of a complex refugial system (Médail & Diadema, 2009; Korkmaz *et al.*, 2010). Determining the characteristics of leading-edge populations and gene flow between different regions within the refugial system therefore requires particular attention.

We note that, since the preparation of this paper, *C. parallelus* has been transferred to a newly established genus, *Pseudochorthippus* (Defaut, 2012). However, to maintain consistency, we use the traditional name *Chorthippus parallelus* until the whole Gomphocerini lineage can be reconsidered on the basis of a larger dataset.

Here, we revisit the *C. parallelus* phylogeographical system, using both previous and newly obtained sequence data of the nuclear *cpnl-1* region, and with particular attention on expanding the sampling and analysis of Anatolian populations. Our first objective is to examine the structure of diversity and differentiation between this region and the others and to investigate the historical and evolutionary processes responsible for such genetic structuring. Second, we aim to examine the potential role of Anatolia as a centre of origin for diversity within *C. parallelus* by including a larger and more geographically diverse collection of Anatolian samples. Third, we investigate patterns of gene flow and likely expansion routes connecting Anatolia with the rest of the current distribution. In particular, we investigate whether the Bosphorus, Dardanelles and Sea of Marmara (hereafter the Turkish Straits system) collectively act as a barrier, using a large dataset of both nuclear DNA (*cpnl-1*) and newly generated mitochondrial DNA (*COI–tRNALeu–COII*) sequences. Finally, we aim to explore the contribution of Anatolian forms to the biodiversity of the Western Palaearctic more generally, based on the evolutionary history of *C. parallelus*.

MATERIALS AND METHODS

Sampling

Specimens were collected from June to August between 2005 and 2009, using several keys to check identification (Bei-Bienko & Mistshenko, 1951). The number of individuals per locality varied due to availability of specimens; those collected were preserved in ethanol for DNA extraction (see Fig. 1 and Appendix S1 in Supporting Information).

Two datasets were generated from both nuclear and mitochondrial genes. The nuclear data included 490 sequences of *cpnl-1*, primarily from Western Europe and Russia from published studies (Cooper & Hewitt, 1993; Cooper *et al.*, 1995) and 559 sequences representing 33 populations from Anatolia, three from Turkish Thrace and two from Bulgaria generated in this work (Appendix S1). The mitochondrial

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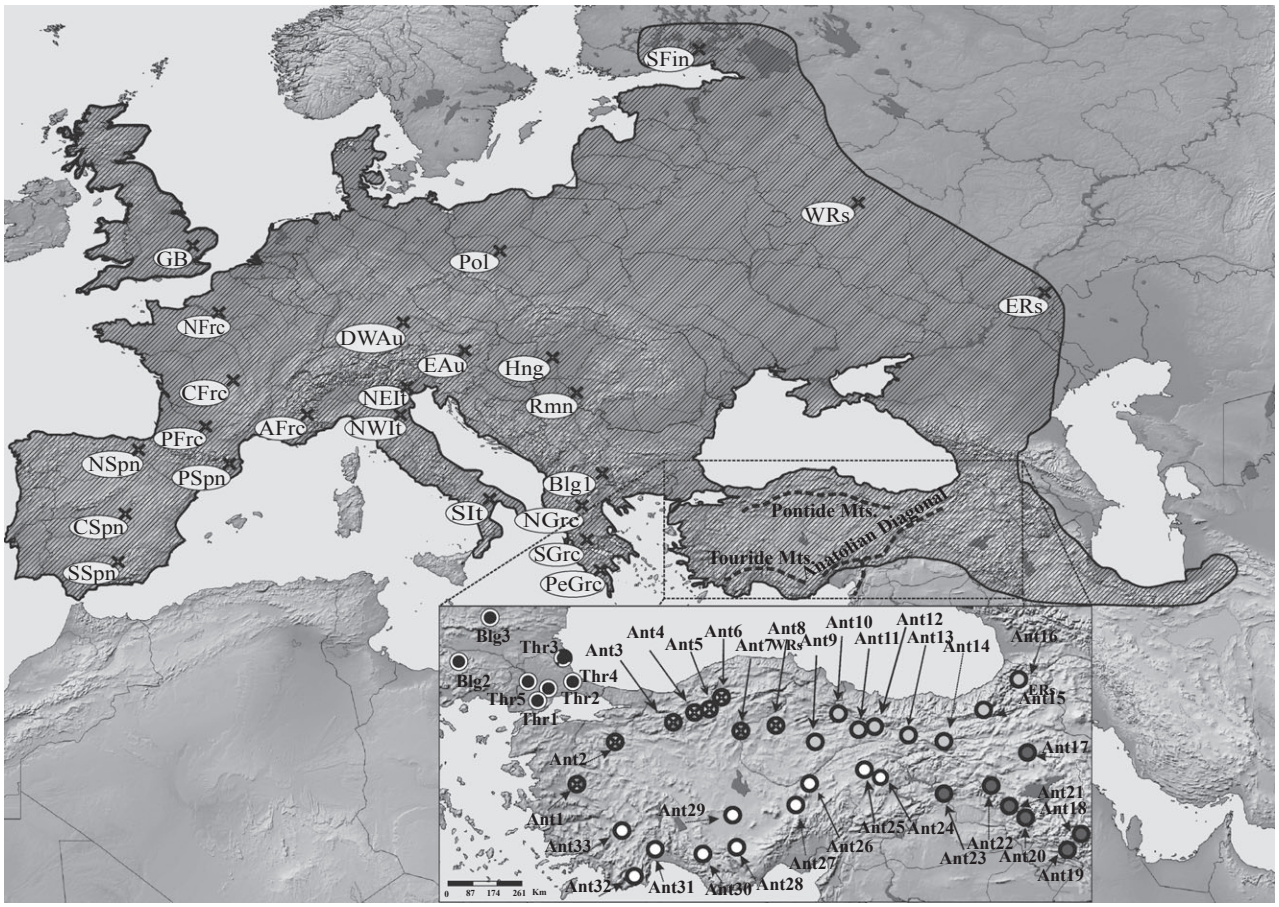

Figure 1 The current known geographical distribution of *Chorthippus parallelus* (hatched area) and sampling localities. Collecting sites from previous studies are indicated by crosses; Anatolian populations were grouped into four regions based on the positions of the Anatolian Diagonal, Taurus and Pontic mountain ranges: NW-Anatolia (Ant1–8); NE-Anatolia (Ant9–17); SE-Anatolia (Ant18–23); and SW-Anatolia (Ant24–33). Detailed information is given in Appendix S1.

dataset generated in this study was only used to test the status of the Turkish Straits system as a barrier, and to investigate the patterns and times of expansion in Anatolian and Thracian populations. It comprised *COI–tRNALeu–COII* sequences from 201 individuals, representing eight populations from north-west Anatolia and five populations from Thrace (Appendix S1).

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from the hind legs of specimens using the Chelex 100 method of Walsh *et al.* (1991). The amplification and single-strand conformation polymorphism (SSCP) analysis of *cpnl-1* (410 bp) followed Korkmaz *et al.* (2010). The mitochondrial *COI–tRNALeu–COII* gene fragment (1433 bp) was amplified using newly designed primers: COIMF (5′-TTGACCCAGCTGGAGGTGGAGAC-3′) and COIIMR (5′-TGATTCCAATAGCAGGAACTGCTC-3′). Amplification was carried out in 50-μL volumes containing 0.5 U of *Taq* polymerase, 5 μL of 10× reaction buffer (100 mM Tris–HCl, pH 8.8, 500 mM KCl and 0.8% Nonidet P-40), 10 pmol of each of the primers, 0.2 mM of each of the four dNTPs, 1.5 mM $MgCl_2$ and 1 μL of DNA template (50–100 ng). PCR cycle conditions were: 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 60.5 °C for 1 min and 72 °C for 30 s; and finally 72 °C for 5 min. Sequencing reactions were carried out in both directions using the primers used in the PCR reactions. The forward and reverse nucleotide sequences were assembled, edited and aligned by eye using CodonCode Aligner 3.5.6 (CodonCode Corporation, Centerville, MA, USA).

Data analysis

Genetic diversity

The sampling locations of *C. parallelus* were divided into 33 regions, based on the geographical proximity of populations and the location of geographical barriers, in Europe (including three Thracian regions), Russia and Anatolia (Fig. 1, Appendix S1). The division of Anatolia into four regions (containing a total 33 of populations) was based on the positions of the Anatolian Diagonal, Taurus Mountains and Pontic Mountains. The Anatolian Diagonal is a mountain

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range running from north-eastern to south-western Anatolia and meeting the Taurus Mountains in the south (Fig. 1). The distribution of genetic diversity across the study area was evaluated in order to detect possible locations of refugia. Nucleotide diversity (π), haplotype diversity (h), the number of segregating/indel sites (S), the number of unique haplotypes and the average number of nucleotide differences (k) were calculated using DnaSP 5 (Librado & Rozas, 2009) and Arlequin 3.5 (Excoffier *et al.*, 2005). We also compared and characterized the distribution of haplotypes containing indel positions. The components of genetic diversity (C_S) and differentiation (C_D) were calculated using contrib 1.02 (Petit *et al.*, 1998).

Genetic structure

The levels of genetic subdivision and gene flow among regions were quantified and tested with the sequence statistic K_{ST} (Hudson *et al.*, 1992). K_{ST} was computed as $K_{ST} = 1 - (K_S / K_T)$, where K_S is the weighted average number of differences between sequences in a region and K_T is the average number of differences between two sequences in the dataset. The correlation of pairwise K_{ST} values with Euclidean geographical distances of regions (Hutchison & Templeton, 1999) was examined using a Mantel test with 10,000 random permutations of the genetic distance matrix, using GenAlEx 6.3 (Peakall & Smouse, 2006).

To detect global genetic structure and to define the most divergent region(s) within the species' range without prior assumptions of region boundaries, a spatial analysis of molecular variance (SAMOVA) based on the proportion of total genetic variance (ϕ-statistics) was carried out in samova 1.0 (Dupanloup *et al.*, 2002). This program requires the number of regions, K, to be given, and we ran samova on our datasets for values of K from two to seven, with 250 simulated annealing processes. The significance levels of ϕ-statistics were estimated by 1000 random permutations. To establish spatial genetic structure in *C. parallelus*, we also performed a discriminant analysis of principal components (DAPC) (Jombart *et al.*, 2010) using the R package adegenet 1.3-1 (Jombart, 2008) (see Appendix S2 for details). We defined a total of 13 indel events across the 29 observed indel positions (see Appendix S1). The clusters generated by the DAPC analysis were superimposed on the maps and the distribution of the indel events was displayed on clusters.

Phylogeography and demographic history

Three datasets of *cpnl-1* were generated: (1) sequence data of all haplotypes; (2) sequence data of the unique haplotypes; and (3) presence and absence of haplotypes, which were coded as '1' and '0', respectively, and analysed under different tree estimating approaches. Network analyses were used to investigate the genealogical relationship between regions using network 4.5.1.6 (Fluxus Technology, Clare, UK; available at http://www.fluxus-technology.com/). The network was calculated by the median-joining (MJ) method ($\varepsilon = 0$) (Bandelt *et al.*, 1999), and a maximum parsimony calculation was subsequently applied. To reduce complexity and evaluate historical demographic expansion events in the network, we also performed an additional analysis using the star-contraction algorithm implemented in the same program.

We then investigated the demographic history of the clusters defined by the DAPC. We first examined Tajima's D (Tajima, 1989), Fu's F_S (Fu, 1997) and the raggedness index (based on the mismatch distribution) (Harpending, 1994) using DnaSP, with 10,000 coalescent simulations, to detect population growth and infer population demographic events. Second, the mismatch distributions and the likely expansion times of the clusters were estimated from the demographic expansion factor statistic tau ($\tau = 2\mu kt$) with 95% confidence intervals using Arlequin, where k is the length of sequences, t is the likely expansion time and μ is the mutation rate per nucleotide. Because there is no estimate of the mutation rate of *cpnl-1*, we used the mutation rate of mitochondrial DNA (mtDNA), which is the proposed average mutation rate of neutral single-copy nuclear DNA in insects (Papadopoulou *et al.*, 2010).

The barrier position of the Turkish Straits system

We used three complementary approaches to test the barrier position of the Turkish Straits system. Thracian and north-west Anatolian groups were created by joining five and eight populations, respectively (Appendix S1). Both *COI–tRNA-Leu–COII* (1433 bp) (GenBank accession numbers: KC107629–KC107654, see Appendix S1 for haplotypes) and *cpnl-1* sequences were used in the analyses. First, the relationships among both mtDNA and *cpnl-1* haplotypes belonging to Thracian and north-west Anatolian groups were separately constructed using network 4.5.1.6. Second, we estimated the likely expansion times of these two groups (Appendix S3). Third, Markov chain Monte Carlo (MCMC) simulations of an isolation-with-migration model were carried out using IMa2 (Hey, 2010) to assess the potential barrier status of the Straits system, divergence time, and rate of gene flow in each direction (see Appendix S3 for more details).

RESULTS

Spatial distribution of genetic diversity

The 1049 *cpnl-1* sequences represented 33 regions across the species' range in Europe, Russia and Anatolia (Fig. 1, Appendix S1). After alignment and trimming, the remaining length of sequences was 314 bp. Overall, 116 variable positions – composed of 87 single-base-pair substitutions, 29 indels, and 40 parsimony informative sites – were observed, defining a total of 188 *cpnl-1* haplotypes (see Appendix S1

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for GenBank accession numbers). Haplotype diversity (h) was 0.848 ± 0.011, ranging from 0.53 to 0.94, and higher than 0.50 in all regions except three [SSpn (0.00 ± 0.00), Blg2 (0.25 ± 0.01) and Blg3 (0.50 ± 0.14); Table 1]. Nucleotide diversity (π) was 0.0090 ± 0.0003 and k was 2.512; π was relatively low for many regions, ranging between 0.000 (SSpn) and 0.039 (ERs), with Blg2 and Blg3 having a score of 0.002 (Table 1). The most common haplotype was Hap2 (found in 18.7% of sequences); 136 haplotypes (72.3% of the overall haplotypes; Table 1) were definable by a single mutation difference and were unique to a single region (Appendix S1). Anatolia, Thrace, Bulgaria and Greece each had unique haplotypes in varying frequencies (see Appendix S1).

The variability among regions in levels of genetic divergence were based mostly on the number of haplotypes detected per region. The contribution of those regions to total diversity is due to the possession of both unique and divergent haplotypes (Fig. 2). Haplotypes found in all Anatolian, Thracian and Russian regions, and most northern European regions, contributed relatively little to the total diversity, whereas negative contributions to the diversity

Table 1 The summary of genetic diversity at regional level in *Chorthippus parallelus*.

Locality	Region	n	S	H	UH	π	h	k
France	CFrc	22	6	7	—	0.005 ± 0.004	0.77 ± 0.08	1.61 ± 0.99
	AFrc	33	17	10	—	0.009 ± 0.005	0.89 ± 0.02	2.72 ± 1.48
	PFrc	70	5	8	—	0.004 ± 0.002	0.80 ± 0.03	1.23 ± 0.79
	NFrc	10	4	3	—	0.007 ± 0.005	0.71 ± 0.09	1.96 ± 1.21
	Total	135	19	13	—	0.006 ± 0.004	0.86 ± 0.01	1.83 ± 1.06
Italy	NWIt	18	14	5	—	0.021 ± 0.012	0.83 ± 0.04	6.35 ± 3.16
	NEIt	12	12	5	1	0.016 ± 0.009	0.79 ± 0.09	4.81 ± 2.53
	SIt	18	16	5	1	0.021 ± 0.012	0.77 ± 0.06	6.44 ± 3.20
	Total	48	18	9	2	0.021 ± 0.011	0.87 ± 0.02	6.43 ± 3.10
Spain	PSpn	50	16	4	1	0.012 ± 0.007	0.53 ± 0.07	3.58 ± 1.85
	NSpn	28	19	9	5	0.021 ± 0.012	0.82 ± 0.05	6.40 ± 3.13
	CSpn	20	13	3	—	0.020 ± 0.011	0.70 ± 0.04	6.19 ± 3.07
	SSpn	12	0	1	—	0.000 ± 0.000	0.00 ± 0.00	0.00 ± 0.00
	Total	110	23	11	6	0.017 ± 0.009	0.72 ± 0.03	5.23 ± 2.55
Northern Europe	DWAu	17	6	7	—	0.006 ± 0.004	0.89 ± 0.04	1.76 ± 1.08
	EAu	24	6	7	2	0.005 ± 0.003	0.76 ± 0.08	1.48 ± 0.93
	GB	8	3	4	1	0.004 ± 0.003	0.79 ± 0.11	1.07 ± 0.79
	Pol	8	3	5	—	0.004 ± 0.003	0.89 ± 0.09	1.32 ± 0.91
	SFin	18	4	4	—	0.005 ± 0.003	0.75 ± 0.06	1.37 ± 0.88
	Hng	9	6	5	2	0.009 ± 0.006	0.89 ± 0.07	2.56 ± 1.51
	Rmn	6	3	4	2	0.006 ± 0.004	0.87 ± 0.13	1.67 ± 1.13
	Total	90	15	20	7	0.006 ± 0.004	0.87 ± 0.03	1.69 ± 1.00
Greece	NGrc	18	13	4	1	0.015 ± 0.009	0.59 ± 0.11	4.48 ± 2.32
	SGrc	18	5	5	1	0.005 ± 0.004	0.82 ± 0.05	1.61 ± 1.00
	PeGrc	15	9	5	3	0.008 ± 0.005	0.70 ± 0.11	2.48 ± 1.42
	Total	51	21	11	5	0.010 ± 0.006	0.76 ± 0.06	3.13 ± 1.65
Bulgaria	Blg1	28	18	9	2	0.013 ± 0.008	0.92 ± 0.03	3.95 ± 2.04
	Blg2	15	2	2	1	0.002 ± 0.002	0.25 ± 0.01	0.50 ± 0.45
	Blg3	13	3	3	1	0.002 ± 0.002	0.50 ± 0.14	0.69 ± 0.56
	Total	56	22	15	4	0.009 ± 0.005	0.80 ± 0.05	2.71 ± 1.46
Thrace	Thr1	11	19	7	2	0.014 ± 0.008	0.89 ± 0.07	4.25 ± 2.28
	Thr2	14	7	6	2	0.005 ± 0.003	0.79 ± 0.09	1.34 ± 0.88
	Thr3	15	7	6	6	0.007 ± 0.004	0.81 ± 0.08	1.98 ± 1.18
	Total	40	27	16	10	0.011 ± 0.006	0.91 ± 0.03	3.35 ± 1.76
Russia	ERs	14	30	6	1	0.040 ± 0.022	0.87 ± 0.05	12.02 ± 5.79
	WRs	14	27	6	—	0.037 ± 0.020	0.89 ± 0.04	11.32 ± 5.47
	Total	28	30	9	1	0.037 ± 0.019	0.88 ± 0.03	11.30 ± 5.29
Anatolia	NWAnt	124	36	63	44	0.015 ± 0.008	0.96 ± 0.01	4.69 ± 2.31
	NEAnt	127	38	31	15	0.019 ± 0.010	0.86 ± 0.02	5.71 ± 2.75
	SEAnt	103	32	20	12	0.015 ± 0.008	0.85 ± 0.02	4.65 ± 2.30
	SWAnt	136	37	49	30	0.020 ± 0.010	0.91 ± 0.02	6.05 ± 2.90
	Total	490	69	129	101	0.019 ± 0.010	0.95 ± 0.01	5.88 ± 2.81

n, number of sequences; S, number of segregating/indel sites; H, number of haplotypes; UH, number of unique haplotypes; π, nucleotide diversity; h, haplotype diversity; k, average number of differences. Full names for regions are given in Appendix S1.

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components were mostly due to Spanish and Balkan haplotypes (Fig. 2).

Genetic structure

The average genetic differentiation (K_{ST}) of all regions was 0.28 and ranged from −0.35 to 0.97 (Appendix S1). Pairwise K_{ST} values between regions showed that the high levels of differentiation were mainly due to remarkably divergent regions of Spain, Italy and Russia, while low levels of differentiation (< 0.1) were commonly observed among Anatolian, Thracian, Balkan and northern European regions, as well as some regions having significant *P*-values (Appendix S1). The Mantel test indicated no significant correlation between K_{ST} and geographical distances ($r^2 = 0.005$; $P > 0.05$).

SAMOVA showed that the F_{CT} value was greatest when all regions were subdivided into two groups: one comprising two Spanish regions (SSpn and PSpn) and the other comprising all other regions ($F_{CT} = 0.491$; Fig. 3). When five groups were included in SAMOVA, the values of F_{CT} and F_{ST} overlapped, and PSpn, SSpn, CSpn, SIt were separated from all remaining regions (Fig. 3). A similar pattern was observed in the pairwise K_{ST} values (Appendix S1).

DAPC analyses indicated a more complex history of the species. The retained PCA components explained 79.9% of the total variance. DAPC partitioned all individuals of *C. parallelus* into four clusters (Fig. 4; see Appendix S2 for a scatter plot), and the posterior probability of assignment for each individual to the correct genetic cluster was 99%, indicating the analysis to be robust (Appendix S2). Clusters A and B divided Anatolia into two parts: individuals from east of the Anatolian Diagonal (58 individuals, 5 haplotypes; Fig. 4a) and those to the west (103 individuals, 32 haplotypes; Fig. 4b). The two remaining clusters have a broad distribution. Cluster C (152 individuals; 25 haplotypes) comprised a mixture of individuals mostly from the southern part of the species' range (Spain, Italy, the Balkans, Russia and western Anatolia) (Fig. 4c), whereas cluster D (736 individuals, 127 haplotypes) included individuals from across the current distribution of the species, mostly from the Anatolian, Thracian, Balkan and northern European regions (Fig. 4d).

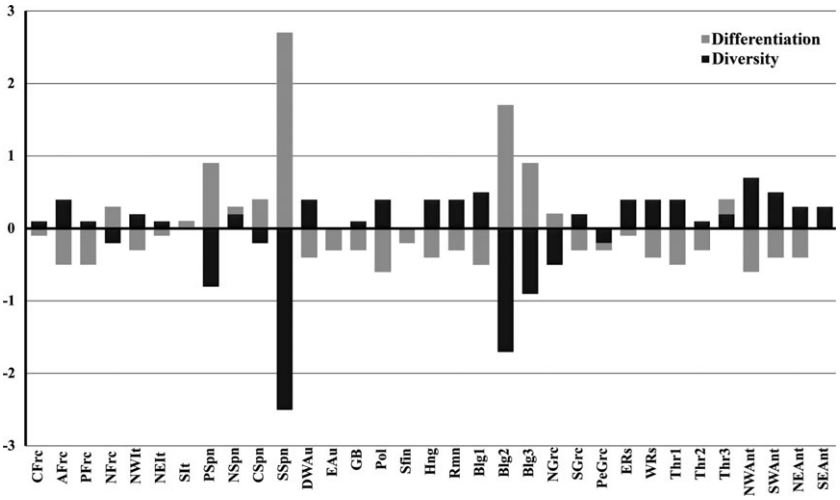

Figure 2 The contribution of each region to the total diversity using *cpnl-1* haplotypes of *Chorthippus parallelus*. The contribution of each region to the total diversity was subdivided into a diversity component (C_S) shown in black, and a differentiation component (C_D) shown in grey bars ($C_T = C_S + C_D$).

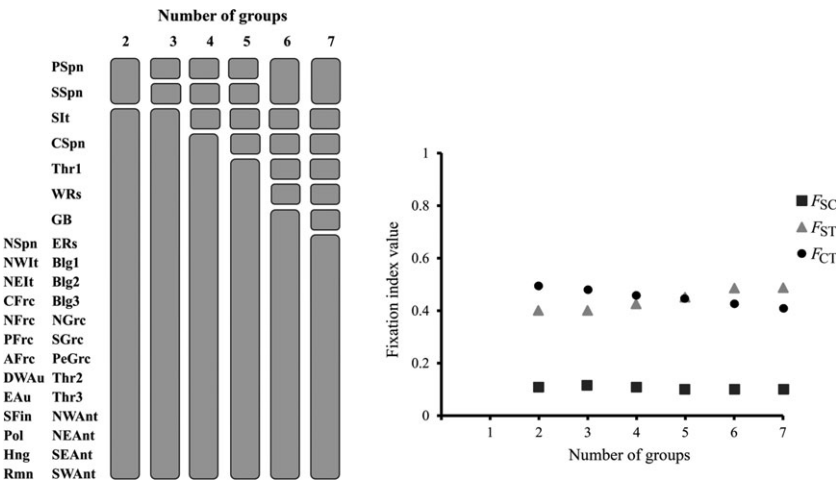

Figure 3 Spatial analysis of molecular variance (SAMOVA) grouping scheme and values of fixation indices obtained for different number of groups of 33 regions of *Chorthippus parallelus*. F_{CT} represents the variation among groups, F_{SC} among regions within groups, and F_{ST} between populations among regions.

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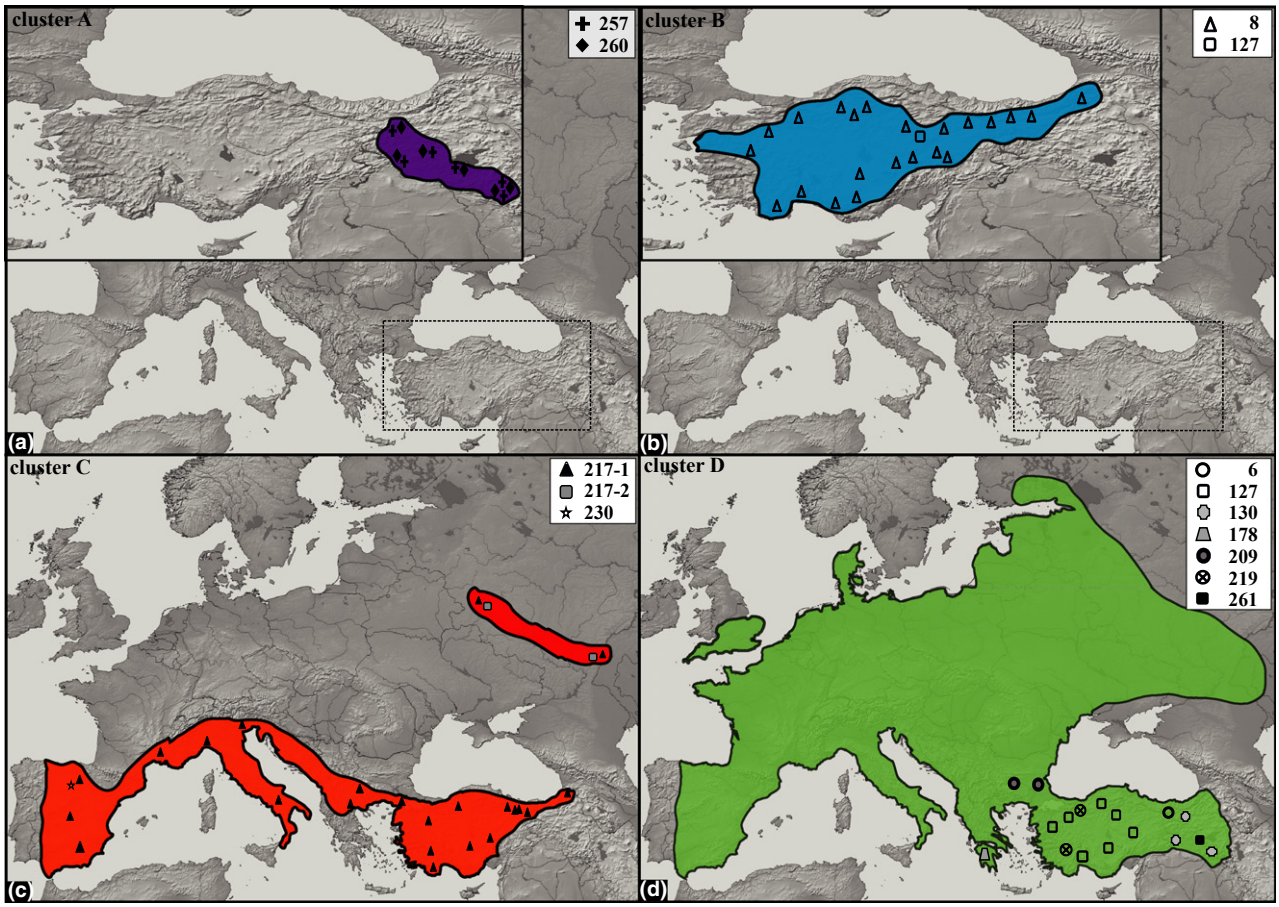

Figure 4 Superimposition of the genetic clusters of *Chorthippus parallelus* generated by the discriminant analysis of principal components (DAPC) on the maps. The numbers specify the site of each indel event. The indel events unique to each cluster and their distribution are indicate by different symbols.

Phylogeography and demographic history

None of the phylogenetic analyses of the three sets of *cpnl-1* haplotype alignments resulted in a well-resolved and robust phylogenetic tree. The lengths of indels varied from 1 to 10 bp (Appendix S1), and the number of indels per sequence showed a decreasing clinal tendency from east to west across the species' range. The highest numbers of total indel positions per sequence was observed in the SEAnt and NEAnt (with total of 21 indel positions) (Appendix S1). The presence of two indel events, at site 257 (GAGA) and at site 260 (A), was observed in all individuals of cluster A, whereas an indel event including three nucleotides at sites 8 (ACT) was detected in all individuals of cluster B (Fig. 4a,b). The most common indel event – at site 217 (AACTT) – was found in all individuals belonging to cluster C, indicating a mostly southern refugial distribution (Fig. 4c). Cluster C also contained two indel events at site 217 (AACGT) in Russia and at site 230 in NSpn. The remaining seven indel events were observed only in certain individuals of cluster D (Fig. 4d). Moreover, five indel events – at sites 6 (A; from NEAnt), 127 (TAT; from NWAnt and SWAnt), 130 (from NEAnt and SEAnt), 219 (CTT; from NWAnt and SWAnt) and 261 (CAGAGA; from SEAnt) – were only found in Anatolia, and two indel events were from Thrace (at site 209 in Thr2 and Thr3) and the Balkans (at sites 178 in PeGrc and 209 in Blg2).

We recovered a high diversity of *cpnl-1* haplotypes, with the network neither dominated by high-frequency alleles nor structured by large divergences between haplotype groups (Fig. 5). The main geographical ranges do not fall into single sections of the network. Anatolian haplotypes (marked in red in Fig. 5) are found throughout the network, closely related to almost all other haplotypes, and most of the low-frequency haplotypes have only been recovered from Anatolia. (See Appendix S3 for the results of neutrality tests and output from the mismatch distribution analysis, including expansion time estimates based on *cpnl-1* sequences for all clusters.) With a generation time of 1 year, the estimated times using the τ statistic of expansion for clusters A–D were 0.041, 0.259, 0.234 and 0.067 Ma, respectively (Appendix S3).

The barrier position of the Turkish Straits system

Prior to the analyses, we investigated several features of mtDNA in order to eliminate the possibility that the data may have included nuclear copies of mitochondrial DNA

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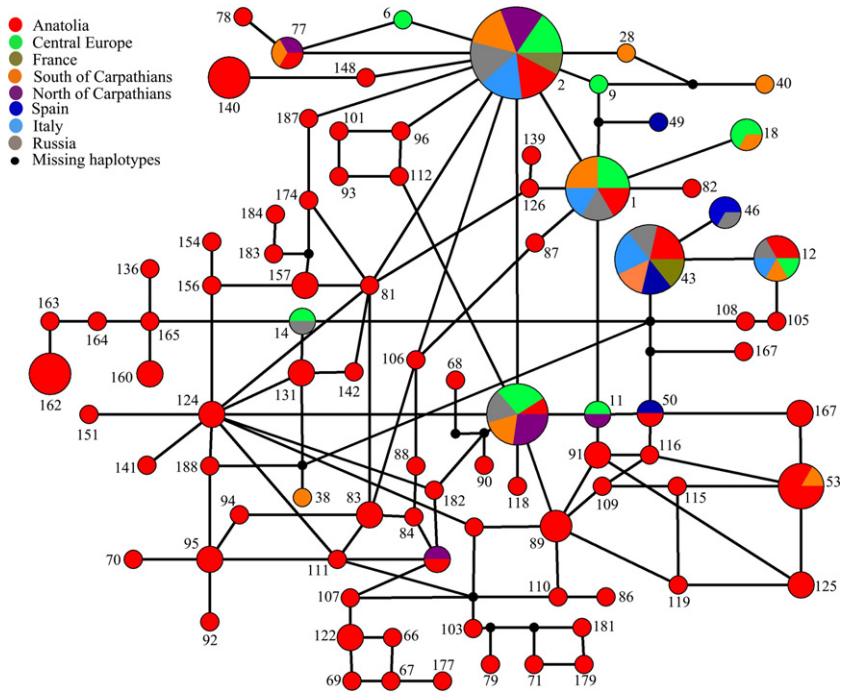

Figure 5 Median-joining network constructed using all *cpnl-1* haplotypes of *Chorthippus parallelus*. Each geographical region is marked with a different colour and numbers correspond to haplotypes in Appendix S1.

(numts). The presence of single peaks in each chromatograph, typical A+T bias observed for insect mtDNA, and the absence of indels and premature stop codons provide good evidence that the aligned sequences correspond to a functional mitochondrial region.

All of the findings of network analyses and demographic statistic tests indicated an undifferentiated population pattern across both sides of the Turkish Straits system, suggesting a recent expansion event or gene flow (see further analysis in Appendix S3). The three IMa2 simulations produced similar results, and all multidimensional peak locations fell within the 95% confidence intervals of the marginal posterior density distributions (Table 2). The estimate of divergence time between Thrace and NWAnt was 0.039 Ma (0.024–0.178) (Table 2, Fig. 6). The effective population size of NWAnt [θ_2 = 281,650 (146,737–519,112)] was estimated to be greater than that of the Thracian group [θ_1 = 158,025 (28,700–315,237)], whereas the ancestral population was significantly smaller [θ_A = 58,025 (100–325,200); Table 2]. The rate of gene flow from NWAnt into Thrace before the Holocene was approximately 20 times greater (m_2 = 2.245) than that in the opposite direction (m_1 = 0.115) (Table 2, Fig. 6).

DISCUSSION

Spatial distribution of genetic diversity

Under the rear/leading edge concept, within-population diversity in glacial ancestral resource regions (or rear edge) is expected to be lower than that of the expanding edge (leading edge) (Hampe & Petit, 2005; Diekmann & Serrão, 2012). The findings of the present study are not consistent with the

Table 2 The results of the isolation-with-migration model for *Chorthippus parallelus*.

	Population size			Migration		Divergence times
	θ_1 (Thrace)	θ_2 (NWAnt)	θ_A (ancestral)	m_1 (Thrace–NWAnt)	m_2 (NWAnt –Thrace)	τ
Estimate						
HiPt	12.6	22.5	4.6	0.115	2.245	0.874
HPD95Low	2.3	11.7	0.1	0.0	0.015	0.542
HPD95High	25.2	41.5	26.0	8.2750	5.325	3.998
Converted						τ (ka)
HiPt	158×10^3	282×10^3	58×10^3	0.0011	0.0224	39.027
HPD95Low	28.7×10^3	147×10^3	0.1×10^3	0.0000	0.0001	24.202
HPD95High	315×10^3	519×10^3	325×10^3	0.0820	0.0532	178.526

Results of analyses used to assess whether the Turkish Straits system forms a barrier between Thracian and north-west Anatolian (NWAnt) populations of *Chorthippus parallelus* by fitting the *COI–tRNALeu–COII* and *cpnl-1* regions to the isolation-with-migration model implemented in IMa2. Parameter estimates are presented for pairwise analysis where effective population size, θ, migration rate, m, and divergence time, τ, are each scaled by the mean mutation rate (see Appendix S3). HiPt, parameter value with the highest posterior probability. HPD95Low/HPD95High, the lower and upper bounds of the 95% highest posterior density confidence interval.

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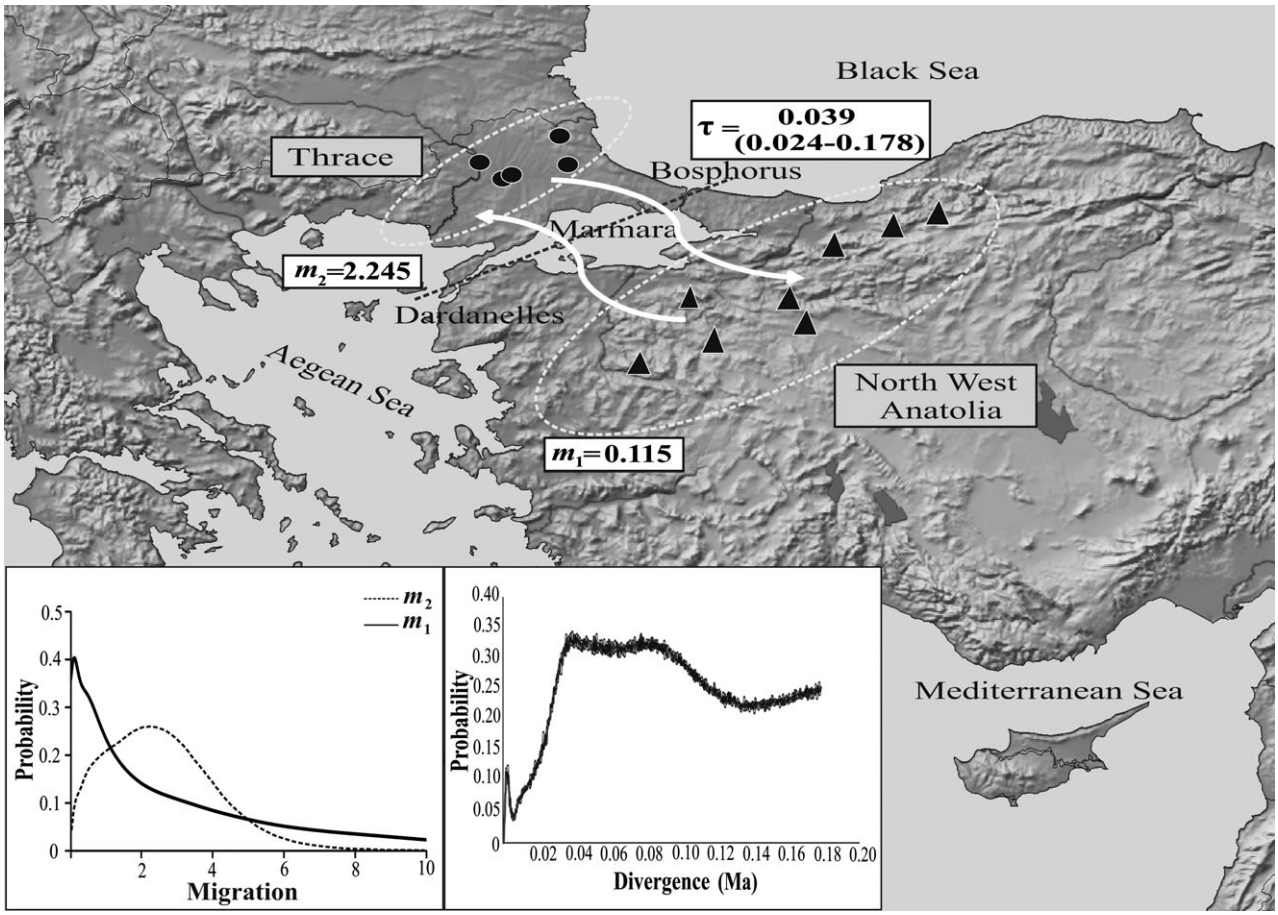

Figure 6 The assessment of the potential barrier status of the Turkish Straits system for *Chorthippus parallelus*, using IMa2. The divergence time (in Ma) and rate of gene flow to each direction are indicated on the map. Parameters are coded as in Table 2. The graphs illustrate the migration rate and divergence time versus their posterior probability plots.

hypothesis that populations at the rear edge are genetically depleted. The meadow grasshopper regions studied here were characterized by high genetic diversity at a regional level, especially in refugia (Table 1), and the results also indicated that most of the total genetic diversity was present within regions (Table 1, Appendix S1). Although haplotype diversity was high, low nucleotide diversity values indicated only small differences between haplotypes (Table 1, Fig. 5). The locations of the regions across the species' range might have triggered the variability of unique haplotypes at regional levels. Indeed, the proportion of unique haplotypes was highest in the southern locations – Anatolia (78.29% of all detected haplotypes), Thrace (62.5%) and Spain (54.55%) (Table 1). We also recorded a relatively high level of nucleotide diversity and average number of nucleotide differences in southern regions (Table 1). The findings suggest that while rear-edge regions of *C. parallelus* are reservoirs of unique genetic variation, this is not the case for the corresponding leading-edge regions.

The spatial distribution of genetic diversity can also provide valuable insights into how rear-edge regions contributed to post-glacial recolonization (Cooper *et al.*, 1995; Hewitt, 1996, 2000; Provan & Maggs, 2012). Italian and Iberian refugial populations of *C. parallelus* have not contributed to the post-glacial recolonization of Europe, probably because of the Pyrenees and Alps acting as significant barriers to dispersal (Cooper & Hewitt, 1993; Lunt *et al.*, 1998). The occurrence of low diversity values in some Spanish (Sspn, PSpn) and Balkan (Blg2 and Blg3) regions (Table 1, Fig. 2) might be explained by their separation from nearby populations by unsuitable habitat; high rates of genetic drift and low rates of gene flow are therefore likely drivers of their higher genetic differentiation (Diekmann & Serrão, 2012). Anatolian regions show a more complex scenario, however, exhibiting the highest level of genetic diversity (Table 1), and the possession of a high number of indel positions (Fig. 4, Appendix S1). The high amount of genetic diversity in Anatolia represents a stable rear edge that has not become genetically depauperate (Cooper *et al.*, 1995; Hewitt, 1996; Dubey *et al.*, 2007; Korkmaz *et al.*, 2010). This pattern has also been reported in several other organisms (Moghaddam *et al.*, 2000; Rokas *et al.*, 2003; Dubey *et al.*, 2007). Anatolia therefore requires a particular attention in terms of conservation and nature protection (Şekercioğlu *et al.*, 2011).

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Geographical structure of genetic differentiation

It is likely that the genetic patterns observed have been greatly influenced by climatic changes associated with the Pleistocene glacial periods, as these are known to influence drift and gene flow, which will combine particularly strongly in the post-glacial recolonization phase (Hewitt, 1999). For wide-ranging species, climate fluctuations may have caused range contractions in some parts of the range and expansions in others (Çıplak, 2004, 2008), which together may have formed the distinct genetic lineages we know today (Hewitt, 2004; Ehrich *et al.*, 2007). In addition to the possible effects of climatic change, topographical structure and mountain chains of a certain region may have led to genetic differentiation. These are consistent with our findings: the analyses related to genetic structuring indicate that populations of *C. parallelus* are not genetically homogeneous across distribution ranges (Figs. 3 & 4, Appendix S1). The formations of clusters A and B suggest that the Anatolian Diagonal acts as an effective barrier to gene flow between the western and eastern regions of Anatolia (Fig. 4a,b). This is also supported by the presence of indel positions specific to both clusters (Fig. 4a,b, Appendix S1). The barrier position of Anatolian Diagonal has also been observed in many taxa in Anatolia (Gündüz *et al.*, 2007, and references therein). On the other hand, the mainly southern distribution of the species together with some Russian individuals appears to represent a relatively old genetic lineage (cluster C in Fig. 4c, Appendix S3). This structuring may have been formed by the direct effect of glacial periods rather than the geographical connections among regions. This is further supported by the presence of the most common indel at site 217 in cluster C and again could be attributed to its old age, possibly resulting from a dispersal wave out of Anatolia (Fig. 4c). This conclusion is consistent with the primary distinction of some southern refugial regions from all others in SAMOVA (Fig. 3) and also the component contributions (Fig. 2). At the same time, the geographical position of cluster C may imply historical connections between Spain, Italy, the Balkans and Anatolia (Fig. 4c). Finally, cluster D consists of the current distribution area of the species, and the lack of any indel events (except in some Anatolian individuals) suggests a relatively new dispersal wave out of Anatolia or the Balkans (Fig. 4d).

Phylogeography, demographic history and the barrier position of the Turkish Straits system

A considerable knowledge on the phylogeography of *C. parallelus* has been accumulated in recent years (Butlin *et al.*, 1991; Cooper & Hewitt, 1993; Cooper *et al.*, 1995; Hewitt, 1996, 1999, 2000; Lunt *et al.*, 1998). These previous studies suggested that the present population in Europe, excluding Spain and southern Italy, was founded by a population dispersing from a Balkan refugium during the last interglacial period. The Spanish and Italian populations are thought to be separate units, originating from their own refugial stocks, arriving in the two peninsulas in an earlier interglacial. Our data are partly consistent with these findings but add more details (see Table 1, Appendix S1). The results support the structuring of Iberia into multiple groups (Figs 3 & 4; Gomez & Lunt, 2007) and a reported relationship between some Spanish and Italian forms (Bella *et al.*, 2007).

The analyses testing the effect of the Turkish Straits system indicated that substantial gene flow between the Balkans and Anatolia has continued since the establishment of these populations (Table 2, Fig. 6). Additional mtDNA data also appear to support this conclusion (Korkmaz, 2011), suggesting the SEAnt populations to be a stock of genotypes involved. The high rate of gene flow from Anatolia to Thrace indicates that Anatolia consistently acted as an important source of diversity for European regions until the Holocene (Table 2, Fig. 6). The marine barrier presented to *C. parallelus* by the Turkish Straits system has of course been influenced greatly by climate cycles. A significant decrease in sea level during the Last Glacial Maximum (Ergin *et al.*, 2007) would have led to a large part of the Sea of Marmara becoming a land mass across which grasshoppers may have dispersed. These results are in agreement with the above suggestions that the Anatolian population contributed to the establishment of central and northern European populations, and in this way the genetic signatures of Anatolia may have extended as far north as Scandinavia before the Holocene (Fig. 4d). This conclusion is entirely in accord with indel distributions (Fig. 4).

Comprehensive data for Anatolian regions have now brought the classic model to a new stage. Hewitt (1996) suggested that the ultimate ancestral region of *C. parallelus* was probably in Anatolia, and our results suggest very strongly that Anatolia is indeed the original source of European populations. We add a number of novel aspects to the classic model of *C. parallelus* phylogeography, including the connectedness of the Balkans and Anatolia despite the apparent marine barrier (Fig. 4, Appendix S1). Multiple expansion and contraction events during the Pleistocene glacial cycles are responsible for the present structuring and distribution of the species, with the formation of the differentiated genetic clusters (Fig. 4). In conclusion, rather than a simple, small, bottlenecked Balkan refugium, we have shown that a larger, more permeable Anatolian centre of origin is a much more realistic model for the Western Palaearctic diversity of *C. parallelus*.

ACKNOWLEDGEMENTS

This study is dedicated to the memory of Godfrey Hewitt (1940–2013), who first advocated *C. parallelus* as a study system, spoke often of the likely importance of Anatolia to European biota, and through mentoring, encouragement and research greatly advanced the study of Eurasian phylogeography as a discipline. Financial support was provided by Cumhuriyet University via research project CUBAP, F-245. A

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part of this project was presented as a PhD thesis of the first author (E.M.K.). We are also grateful to the Council of Higher Education (Turkey) for providing a bursary of 6 months to E.M.K. to carry out a part of his PhD research project in the bioinformatics laboratory of Dave Lunt (University of Hull, UK). We are also grateful to Günter Köhler (Friedrich Schiller University, Jena, Germany) and Dragan Chobanov (Bulgarian Academy of Science, Sofia, Bulgaria) for supplying some of the specimens studied. Our thanks also go to Sarp Kaya, Mahir Budak and Mahir Yıldırım for their help and companionships during field studies. We are also indebted to the anonymous referees for their critical comments on earlier versions of the manuscript.

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SUPPORTING INFORMATION

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

Appendix S1 Detailed description of study material and defined haplotypes and pairwise K_{ST} estimates.

Appendix S2 Detailed description of DAPC analysis in R package.

Appendix S3 Results of demographic history and IMa2 analyses.

BIOSKETCH

E. Mahir Korkmaz, an assistant professor at the Cumhuriyet University, is interested in the systematics and molecular evolution of Western Palaearctic species. In particularly, he is focused on Anatolian historical biogeography and mitochondrial genome evolution in insects. The research interests of the coauthors include molecular phylogeny, phylogeography, bioinformatics and biogeography.

Author contributions: a core team (E.M.K., B.Ç. and H.H.B.) conceived the principal ideas and the main structure of the manuscript and led the writing; E.M.K., B.Ç. and N.D. collected the specimens and data; D.L. helped with the analyses and writing.

Editor: Melodie McGeoch

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