1    Original Article

2    **The contribution of Anatolia to European phylogeography: the centre of origin for the**

3    **meadow grasshopper *Chorthippus parallelus***

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15    **Short running header** Phylogeography of the meadow grasshopper *Chorthippus parallelus*

16    **Abstract**

17    **Aim** *Chorthippus parallelus* is one of the classic model systems for studying genetic structure

18    and phylogeography of the Western Palaearctic. Here, we investigate the regional genetic

19    differentiation of *C. parallelus* and evaluate historical and evolutionary processes responsible

20    for such genetic structuring; test the nature of the Marmara water body as barriers to dispersal,

21    and explore the contribution of Anatolian populations to the biodiversity of the West

22    Palaearctic including its likely expansion routes.

23    **Location** West Palaearctic.

24    **Methods** We have incorporated sequence data from detailed sampling of the

25    phylogeographically important Anatolian region using both previous and newly obtained

26    sequence data of the nuclear *cpnl-1* and the mitochondrial *COI-tRNAleu-COII*. A total of

27    1049 sequences of *cpnl-1* from 33 regions were analysed to investigate the genetic diversity,

28    genetic structuring and phylogeography of *C. parallelus* across its distribution range. The

29    mtDNA region was additionally utilised to test the barrier position of the Marmara water

30    body.

31    **Results** The analyses revealed that not all southern refugial populations of *C. parallelus* have

32    contributed equally to the postglacial recolonization of Europe. Four genetic clusters across

33    the species range were recovered: Cluster A (east part of the Anatolian Diagonal), Cluster B

34    (west part of the Anatolian Diagonal), Cluster C (Spain, Italy, Southern Balkans, west part of

35    Anatolia and Russia), and Cluster D (current distribution range of the species). The Marmara

36    water body has been a weak barrier to dispersal by *C. parallelus*, allowing gene flow from

37    Anatolia to the Balkans.

38    **Main conclusions** The current patterns of genetic structuring of *C. parallelus* were best

39    explained by multiple expansion and contraction events. Anatolia has been well connected to

40    the Balkans, contributing genetically to the establishment of central and northern European

41    populations, and its expansion prior to the Holocene will have extended as far north as

42    Scandinavia. The Anatolian refugium is suggested to be the centre of origin for west

43    Palaeoarctic *C. parallelus* diversity rather than a Balkan refugium.

## Introduction

Phylogeographic studies of Western Palaearctic lineages have allowed several important generalisations concerning the history of temperate species (Hewitt, 1996, 2000; Taberlet *et al.,* 1998). One such is that the Pleistocene glacial cycles have had significant effects on the present distribution of many species as the biodiversity of northern areas are 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, and the Balkans 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). Three common routes of recolonization from southern refugia to northern areas have been recognized (Hewitt, 1999); however additional routes may also have played a role (Taberlet *et al.,* 1998; Rokas *et al.,* 2003; Schmitt, 2007).

Although not rigorously tested, Anatolia has also been suggested as an important glacial refugium (Hewitt, 1996; Rokas *et al.,* 2003; Çıplak, 2004, 2008; Korkmaz *et al.,* 2010; Ansell *et al.,* 2011). The geographic location of Anatolia together with its complex paleogeographic history, heterogeneous topography and climate contributes to the high levels of endemism and global importance of its biodiversity (Şekercioğlu *et al.,* 2010). Albeit data is still limited, studies focusing on several lineages reveal 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). Anatolia, however, has been underrepresented in regional phylogeographic studies with specifically Anatolian lineages, or representatives of Anatolian populations for wide-ranging forms, rarely, inadequately, or only recently being included. With the effects of the climatic shifts of Pliocene/Pleistocene, vertical range changes may be expected depending on the heterogeneous topography. In particular during the warm interglacials the populations

72    preferring cold conditions would have been isolated and possibly diverged on top of high

73    mountains or ʻsky islandsʼ and this feature may suggest a complex refugial system for

74    Anatolia (Çıplak, 2004, 2008).

75    The meadow grasshopper *Chorthippus parallelus* (Zettersted, 1821) has been a key example

76    of phylogeographic structuring and indeed gives its name to one of Hewitt's three refugial

77    expansion models (Hewitt, 1999). Phylogeographic studies of *C. parallelus* have utilized both

78    mitochondrial DNA and nuclear loci and suggest three major genomic units in Iberia,

79    Calabria (Southern Italy), and the populations in remaining range of the species (Cooper *et*

80    *al.,* 1995; Lunt *et al.,* 1998). Besides the acceptance that the present European range has

81    originated by postglacial expansion from the Balkans, Hewitt (1996) also suggested that

82    Anatolian populations may have survived the glacial cycles and contributed to the

83    repopulation of Europe. Some Anatolian specimens were included in the study by Cooper *et*

84    *al.* (1995), but this sampling was insufficient to properly represent either genetic diversity or

85    geographic fragmentation of the species within Anatolia (Korkmaz *et al.,* 2010). The

86    implication of Hewitt (1996) that Anatolia could be the ultimate origin of the species has

87    remained therefore largely untested. An investigation of the relationship among Anatolian

88    populations may contribute to our understanding of the origins of *C. parallelus* diversity

89    during the expansion periods. The other issue to be considered is the direction of range

90    change during either cold or warm periods. It is generally accepted that during interglacial

91    periods the northern edge of the population was the leading or expanding edge and the

92    southern edge was the eroding or rear edge, while the reverse directions were the case in cold

93    periods (Çıplak, 2004; Hampe & Petit, 2005). However some assumptions of the rear

94    edge/leading edge hypothesis appear to be in conflict with the characteristics of a complex

95    refugial system (Médail & Diadema, 2009; Korkmaz *et al.,* 2010). Therefore, determining the

96 characteristics of leading edge populations and gene flow between different regions within the

97 refugial system requires particular attention.

98 We note that since the preparation of this paper, *C. parallelus* has been transferred to a newly

99 established genus *Pseudochorthippus* (Defaut, 2012) to maintain consistency we use the

100 traditional name *Chorthippus parallelus* until the whole Gomphocerini lineage can be

101 reconsidered on the basis of a larger dataset.

102 Here we revisited the *C. parallelus* phylogeographic system, using both previous and newly

103 obtained sequence data of the nuclear *cpnl-1* region, and with particular attention to expand

104 the sampling and analysis of its Anatolian populations. Our first objective is to examine the

105 structure of diversity and differentiation between this region and the others and to investigate

106 the historical and evolutionary processes responsible for such genetic structuring. Second, we

107 aim to examine the potential role of Anatolia as a centre of origin for diversity within *C.*

108 *parallelus* by including a larger and more geographically diverse collection of Anatolian

109 samples. Thirdly, we investigate patterns of gene flow and likely expansion routes connecting

110 Anatolia with the rest of the current distribution. In particular, we investigate whether the

111 Bosphorus and Dardanelles, and Marmara Sea (hereafter is called the Marmara water body)

112 acts as a barrier using a large dataset of both nuclear DNA (*cpnl-1*) and newly generated

113 mtDNA (*COI-tRNALeu-COII*) sequences. Finally, we aim to explore the contribution of

114 Anatolian forms to biodiversity of West Palaearctic more generally based on the evolutionary

115 history of *C. parallelus.*

116

117 **Materials and Methods**

118 **Sampling**

119 The species used in this study is not endangered or protected, nor are the sampling locations;

120 therefore no specific permits were required. Specimens were collected from June to August

121 between 2005 and 2009, using several keys to check identification (Bei-Bienko &

122 Mistshenko, 1951). The number of individuals per locality varied due to availability of

123 specimens and those collected were preserved in EtOH for DNA extraction (see Fig. 1 &

124 Appendix S1 in Supporting Information for sampling localities of both previous and new

125 datasets).

126 Two different datasets were generated from both nuclear and mitochondrial genes. The

127 nuclear data included *cpnl-1* sequences of 248 individuals primarily from W. Europe and

128 Russia from published studies (Cooper & Hewitt, 1993; Cooper *et al.,* 1995); and that of 558

129 individuals representing 33 populations from Anatolia, three from Thracian Turkey and two

130 from Bulgaria generated in this work (Appendix S1). The mitochondrial data set generated in

131 this study was used in testing the barrier statues of the Marmara water body and investigating

132 expansion patterns and times of Anatolian and Thracian populations. It comprised of *COI-*

133 *tRNALeu-COII* sequences from 201 individuals representing eight populations from northwest

134 Anatolia and five populations from Thracia (Appendix S1). The mtDNA region utilised in this

135 study is not comparable with the region that was used in previous studies (Lunt *et al.,* 1998).

136 **DNA extraction, PCR and sequencing**

137 Total genomic DNA was extracted from the hind leg of specimens using the Chelex-100

138 method described by Walsh *et al.* (1991). The amplification and single strand conformation

139 polymorphism (SSCP) analysis of *cpnl-1* (410 bp) were carried out by the method outlined in

140 Korkmaz *et al.* (2010). The mitochondrial *COI-tRNALeu-COII* gene fragment (1433 bp) was

141 amplified using newly designed primers: COIMF (5'-TTGACCCAGCTGGAGGTGGAGAC-

142 3') and COIIMR (5'-TGATTCCAATAGCAGGAACTGCTC-3'). Amplification was carried

143 out in 50-µL volumes containing 0.5 U of *Taq* polymerase, 5 µL of 10 × reaction buffer (100

144 mM Tris-HCl, pH 8.8, 500 mM KCl, and 0.8% Nonidet P-40), 10 pmol of each of the primers,

145 0.2 mM of each of the four dNTPs, 1.5 mM $MgCl_2$, and 1 µL of DNA template (50-100 ng).

146 PCR cycle conditions were: 94 $^{\circ}$C, 5 min; 35 × (94 $^{\circ}$C, 1 min; 60.5 $^{\circ}$C, 1 min; 72 $^{\circ}$C, 30 s);

147 72 $^{\circ}$C, 5 min. Sequencing reactions were carried out in both directions using the same primers

148 as in PCR reactions. The forward and reverse nucleotide sequences were assembled, edited

149 and aligned by eye using the CODONCODE ALIGNER 3.5.6 (CodonCode Corporation).

150 **Data analysis**

151 **Genetic diversity**

152 The sampling locations of *C. parallelus* were divided into 33 regions, based on the geographic

153 proximity of populations and the location of geographic barriers, in Europe (including three

154 Thracian regions), Russia and Anatolia (Fig. 1 & Appendix S1). Anatolia was also divided

155 into four regions (including a total 33 of populations) considering the positions of the

156 Anatolian Diagonal as well as Taurus and Pontids Mts ranges. The distribution of genetic

157 diversity across the study area was evaluated in order to detect signals for refugial position.

158 Nucleotide diversity ($\pi$), haplotype diversity ($h$), number of segregating/indel sites ($S$),

159 number of unique haplotypes and average number of nucleotide differences ($k$) were

160 calculated using DNASP 5 (Librado & Rozas, 2009) and ARLEQUIN 3.5 (Excoffier *et al.,*

161 2005). We also compared and characterised the distribution of haplotypes containing indel

162 positions. The components of genetic diversity ($C_S$) and differentiation ($C_D$) were also

163 calculated using the computer software CONTRIB 1.02 (Petit *et al.,* 1998), to estimate the

164 contribution of each region to genetic diversity and genetic divergence.

165 **Genetic structure**

166 The levels of genetic subdivision and gene flow among regions were quantified and tested

167 applying the sequence statistic, $K_{ST}$ (Hudson *et al.,* 1992). $K_{ST}$ was computed as $K_{ST}$ =1−

168 ($K_S/K_T$); where $K_S$ = weighted average number of differences among sequences in a region

169 and $K_T$ = average number of differences between two sequences in the dataset. The pairwise

170 $K_{ST}$ values against Euclidian geographic distances of regions (Hutchison & Templeton, 1999)

171  was tested using the Mantel option with 10.000 random permutations of genetic distance

172  matrix in the GENALEX 6.3 (Peakall & Smouse, 2006).

173  In order to detect global genetic structure and to define the most divergent region(s) within

174  the species range without prior region boundary assumptions, a spatial analysis of molecular

175  variance (SAMOVA) based on the proportion of total genetic variance ($\Phi$-statistics) was

176  carried out by SAMOVA 1.0 (Dupanloup *et al.,* 2002). This program requires the number of

177  *K* and we ran SAMOVA on our datasets on different *K*, ranging from 2 to 7, with 250

178  simulated annealing processes. Significance levels of $\Phi$-statistics were estimated by 1000

179  random permutations. To establish spatial genetic structure in *C. parallelus*, we also

180  performed a discriminant analysis of principal components (DAPC) (Jombart *et al.,* 2010).

181  See Appendix S2 for a more detailed explanation of the DAPC analysis using the R package

182  ADEGENET 1.3-1 (Jombart, 2008). Twenty nine indel positions were also observed with

183  many consisting of two or more sites. We therefore defined a total of 13 indel events (see

184  Appendix S1). The distribution of the indel events were superimposed on the clusters

185  generated by the DAPC analysis.

186  **Phylogeography and demographic history**

187  Three datasets were generated: (i) sequence data of all haplotypes; (ii) sequence data of the

188  unique haplotypes; and (iii) presence and absence of haplotypes which were coded as (1) and

189  (0) respectively and analysed under different assumptions by application of maximum

190  likelihood, maximum parsimony, Bayesian inference and minimum evolution (NJ)

191  approaches. We also carried out network analyses to investigate the genealogical relationship

192  between regions. We constructed a haplotype network using NETWORK 4.5.1.6 (available at

193  http://www.fluxux-technology.com). Network was calculated by the median-joining (MJ)

194  method ($\varepsilon = 0$) (Bandelt *et al.,* 1999) and a subsequent maximum parsimony calculation was

195  applied. To reduce complexity and evaluate historic demographic expansion events in the

196 network, we also performed an additional analysis using star contraction algorithm
197 implemented in the same program.

198 We then investigated the demographic history of the clusters defined by the DPCA. We firstly
199 examined Tajima's $D$ (Tajima, 1989), Fu's $Fs$ (Fu, 1997) and the raggedness index (based
200 on the mismatch distribution) (Harpending, 1994), using DNASP, with 10.000 coalescent
201 simulations, to detect population growth and infer population demographic events. Second,
202 the mismatch distributions and the likely expansion times of the clusters were estimated from
203 demographic expansion factor static Tau ($\tau = 2\mu kt$) with 95% confidence intervals by using
204 ARLEQUIN 3.5, where k is the length of sequences and $\mu$ is the mutation rate per nucleotide.
205 Because there is no estimate on mutation rate of the non-coding $cpnl-1$, we utilised the same
206 mutation rate used in mtDNA which is the proposed average rate of neutral single copy
207 nuclear DNA in insect (Papadopoulou $et\ al.,$ 2010).

208 **The barrier position of the Marmara water body**
209 We used three complementary approaches to test the barrier position of the Marmara water
210 body. Thracian and North-West Anatolian groups were created by inclusion of five and eight
211 populations respectively (Appendix S1). The $COI-tRNALeu-COII$ (1433 bp) (GenBank
212 accession numbers: KC107629 - KC107654, see Appendix S1 for haplotypes) and $cpnl-1$
213 sequences were utilised in the analyses. First, the relationships among both mtDNA and $cpnl-$
214 $1$ haplotypes belonging to Thracian and North-West Anatolian groups were separately
215 constructed using NETWORK 4.5.1.6. Second, we estimated the likely expansion times of
216 these two groups. Third, Markov Chain Monte Carlo (MCMC) simulations of the Isolation
217 with Migration model using the IMA2 software (Hey, 2010) were carried out to assess the
218 potential barrier status of the water body, splitting time, and rate of gene flow to each
219 direction. See Appendix S3 for a more detailed explanation of the IMA2 analysis.

220

221    **Results**

222    **Spatial distribution of genetic diversity**

223    The 1049 *cpnl-1* sequences represented 33 regions across the species range in Europe, Russia

224    and Anatolia (Fig. 1 & Appendix S1). After alignment and trimming, the remaining length of

225    sequences was 314 bp. Overall, 116 variable positions, composed of 87 single bp

226    substitutions, 29 indels, and 40 parsimony informative sites were observed defining a total of

227    188 *cpnl-1* haplotypes (GenBank accession numbers were given in Appendix S1). *h* was

228    $0.848 \pm 0.011$ and generally higher than 0.50 in all regions ranging from 0.53 to 0.94 except

229    in three regions (SSpn, Blg2 and Blg3) (Table 1). $\pi$ was $0.0090 \pm 0.0003$ and *k* was 2.512. $\pi$,

230    in contrast, was relatively low for many regions, ranging between 0.000 (SSpn) and 0.039

231    (ERs) with Blg2 and Blg3 having a score of 0.002 (Table 1). The most common haplotype

232    was Hap_2 (found in 18.7% of sequences). The 136 haplotypes (72.3% of the overall

233    haplotypes, Table 1) were definable with a singleton mutation difference and were unique to a

234    single region (Appendix S1). Each region of Anatolia, Thracia, Bulgaria and Greece had

235    unique haplotypes in varying frequencies (Appendix S1). The frequencies of the shared

236    haplotypes were presented in Appendix S1.

237    The variability levels of genetic divergence among regions were based mostly on the number

238    of haplotypes detected per region. The contribution of those regions to total diversity is due to

239    possession of both unique and divergent haplotypes (Fig. 2). Haplotypes found in all

240    Anatolian, Thracian, Russian, and most northern European regions provided relatively low

241    positive contribution to total diversity while negative contribution of the diversity components

242    was mostly due to Spanish and Balkans haplotypes (Fig. 2).

243    **Genetic structure**

244    Average genetic differentiation ($K_{ST}$) of all regions was 0.28 and ranged from -0.35 to 0.97

245    indicating a variable genetic differentiation among regions (Table 2). Pairwise $K_{ST}$ values

246    between regions showed that the high levels of differentiation were mainly due to remarkably

247    divergent regions of Spain, Italy and Russia. On the other hand, low levels of differentiation

248    (< 0.1) were commonly observed among Anatolian, Thracian, Balkans and northern European

249    regions as well as some regions having significant $P$ values (Table 2). The Mantel test

250    indicated no significant correlation between $K_{ST}$ and geographic distances of the species range

251    ($R^2 = 0.005$, $P > 0.05$).

252    We applied successive SAMOVA analyses to the data matrix of regions to see possible

253    structuring in a geographic basis (Fig. 3). The results showed that $F_{CT}$ value was the highest

254    when all regions were subdivided into two groups; two Spanish regions (SSpn-PSpn) and all

255    other remaining regions ($F_{CT} = 0.491$, Fig. 3). When five groups were included in SAMOVA,

256    $F_{CT}$ and $F_{ST}$ values overlapped and PSpn, SSpn, CSpn, SIt were separated from all remaining

257    (Fig. 3). A similar pattern was observed in the pairwise $K_{ST}$ values [from 0.21 (SSpn-CSpn) to

258    0.97 (SSpn-Blg2) for SSpn, from 0.23 (PSpn-CSpn) to 0.77 (PSpn-PFrc) for PSpn, from 0.12

259    (CSpn-NWIt) to 0.69 (CSpn-PFrc) for CSpn and from 0.07 (SIt-NSpn) to 0.72 (SIt-PFrc) for

260    SIt, but not among them (Table 2)].

261    The result of DAPC analyses indicated a more complex history of the species. The retained

262    PCA components explained 79.9% of the total variance observed. The DAPC analysis

263    partitioned the all individuals of *C. parallelus* into four clusters (Fig. 4 & see Appendix S2 for

264    a scatterplot graph). Moreover, the posterior probability of assignment for each individual to

265    the correct genetic cluster was 99%, indicating a high robustness of the analysis (Appendix

266    S2). The Cluster A and B divided Anatolia into two parts, of which one corresponded to

267    individuals from east part of the Anatolian Diagonal (58 individuals, 5 haplotypes; Fig. 4a);

268    the other corresponded to west part of Anatolian Diagonal (103 individuals, 32 haplotypes;

269    Fig. 4b). On the other hand, the remaining two clusters displayed relatively a broad

270    distribution. Cluster C (152 individuals; 25 haplotypes) was comprised a mixture of

271  individuals mostly from southern range of the species (Spain, Italy, Balkans, Russia and

272  western Anatolia) (Fig. 4c); while Cluster D (736 individuals, 127 haplotypes) corresponded

273  to the current distribution of the species with individuals mostly from Anatolian, Thracian,

274  Balkans, and northern European regions (Fig. 4d).

275  **Phylogeography and demographic history**

276  None of our diverse phylogenetic analyses of the three sets of *cpnl-1* haplotype alignments

277  resulted in a well-resolved phylogenetic tree, which is not unusual for population level

278  diversity. The length of indels exhibited a great variation from one to 10 bp in the fragment

279  (Appendix S1). Number of indels per sequence showed a clinal tendency from east to west

280  across the species range. The highest numbers of total indel positions per sequence was

281  observed in the SEAnt and NEAnt (with total of 21 indel positions) (Appendix S1). The

282  presence of two indel events at site 257 (GAGA), and at site 260 (A) was observed in all

283  individuals of Cluster A, while an event including three nucleotides at sites 8 (ACT) was

284  detected in all individuals of Cluster B (Fig. 4ab). The most common indel event at site 217

285  (AACTT) was found in all individuals belong to Cluster C indicating mostly a southern

286  refugial distribution (Fig. 4c). Cluster C also contained two indel events at site 217 (AACGT)

287  in Russia and at site 230 in NSpn. On the other hand, the remaining seven indel events were

288  observed only in certain individuals of Cluster D (Fig. 4d). Moreover, five indel events (at

289  sites 6 (A; from NEAnt), 127 (TAT; from NWAnt and SWAnt), 130 (from NEAnt and

290  SEAnt), 219 (CTT; from NWAnt and SWAnt) and 261 (CAGAGA; from SEAnt) were only

291  from Anatolia, and two indel events were from Thracia [at site 209 (from Thr2 and Thr3)] and

292  Balkans [at sites 178 (from PeGrc) and 209 (A; from Blg2)].

293  We also investigated network approaches to the data and present a median-joining haplotype

294  network in Fig. 5. The figure shows the high diversity of *cpnl-1* haplotypes recovered, and

295  reveals that the network is not dominated either by high-frequency alleles nor structured by

296 large divergences between haplotype groups. The main geographic ranges, illustrated in the

297 figure by separate colours, do not fall into single sections of the network indicating

298 inheritance of haplotypes that are not necessarily each others' closest relatives. Anatolian

299 haplotypes (red) are found throughout the network, closely related to almost all other

300 haplotypes, and most of the low-frequency haplotypes have only been recovered from

301 Anatolia. Results of neutrality tests and output from the mismatch distribution analysis

302 including expansion time estimates based on *cpnl-1* sequences for all clusters are provided in

303 of Appendix S3. With a generation time of one year, the estimated times using $\tau$ statistic of

304 expansion for Cluster A-D respectively were 0.041, 0.259, 0.234 and 0.067 mya (Appendix

305 S3).

306 **The barrier position of the Marmara water body**

307 Prior to the analyses, we investigated several features of the mtDNA in order to eliminate the

308 possibility that the data may have included nuclear copies of mitochondrial DNA (numts).

309 The presence of single peaks in each chromatograph, typical A+T bias observed for insect

310 mtDNA, and the absence of indel and premature stop codons provide good evidence that the

311 aligned sequences correspond to a functional mitochondrial region.

312 All of the findings of network analyses and demographic statistic tests indicated an

313 undifferentiated population pattern across both sides of the Marmara water body indicating a

314 recent expansion event or gene flow (See Appendix S3 for a more detailed comparison of the

315 findings of network analyses and demographic statistic tests). The three IMA simulations

316 produced similar results and all multidimensional peak locations fell within the 95%

317 confidence intervals of the marginal posterior density distributions (Table 3). The estimates of

318 divergence time for the Thracia-NWAnt were $\tau_{\text{Thracia-NWAnt}} = 0.039$ mya (0.024-0.178)

319 (Table 3 & Fig. 6). In addition to the divergence time, we estimated the effective population

320 size and the rate of gene flow between groups to test the possible effects of the current barrier

321　to population parameters. The effective population size of the NWAnt [ $\theta_2$ = 281650

322　(146737-519112)] was greater than that of the Thracian group [ $\theta_1$ = 158025 (28700-

323　315237)], whereas the ancestral population was significantly smaller [ $\theta_A$ = 58025 (100-

324　325200); Table 3]. The rate of gene flow from the NWAnt to the Thracia was approximately

325　20 times greater ($m_2$ = 2.245) than that in the opposite direction ($m_1$ = 0.115) (Table 3 & Fig.

326　6).

327

328　**Discussion**

329　We observe a remarkable genetic diversity and genetic structuring of *C. parallelus* in

330　Anatolia. This supports previous studies suggesting Anatolia either as a centre of diversity or

331　a glacial refugium (Hewitt, 1999; Çıplak, 2008; Çıplak *et al.,* 2010; Ansell *et al.,* 2011;

332　Sekercioğlu *et al.,* 2011). The evidences presented here are consistent with Anatolia as the

333　most diverse of all European regions in terms of haplotype and nucleotide diversity (Table 1)

334　as well as indel events (Fig. 4 & Appendix S1); although increased sampling of the whole

335　distribution range is needed for more quantative comparisons. Multiple expansion and

336　contraction events during the Pleistocene glacial cycles appear to be responsible for the

337　present structuring and distribution of the species, with the formation of the differentiated

338　genetic clusters (Fig. 4). Our data also reveals that Anatolian populations have had persistent

339　contact and interaction with European populations, specifically with exchange between

340　Balkans populations via Thracia and western Anatolia. These exchanges were also supported

341　by confirmation that the Marmara water body was not a significant barrier to *C. parallelus*

342　until the Holocene allowing a high level of gene flow from Anatolia to the Balkans (Table 3

343　& Fig.6). The implications of our results are discussed in detail below.

344　**Spatial distribution of genetic diversity**

345 According to the rear/leading edge concept, within/between population diversity in glacial

346 ancestral resource regions (or rear edge) are expected to be higher than that of expanding edge

347 (or leading edge) (Hampe & Petit, 2005; Diekman & Serrão, 2012). The findings of the

348 present study are not consistent with the main hypothesis that small populations isolated in the

349 rear edge are genetically depleted. The meadow grasshopper regions studied here were

350 characterized by high genetic diversity at the regional levels especially in refugia (Table 1)

351 and the results also indicated that most of the total genetic diversity was present within

352 regions (Table 1 & Appendix S1). Although haplotype diversity was high, low nucleotide

353 diversity values indicated only small differences between haplotypes (Table 1 & Fig. 5). The

354 locations of the regions across the species range might have triggered the variability of unique

355 haplotypes at regional levels. Indeed, the percent of unique haplotypes was highest in the

356 southern locations—Anatolia (78.29% of all detected haplotypes), Thracia (62.5%) and Spain

357 (54.55%) (Table 1). We also recorded a relatively high level of nucleotide diversity and

358 average number of nucleotide differences in southern regions (Table 1). The findings

359 suggested that while rear edge regions of *C. parallelus* are reservoirs of unique genetic

360 variation, this is not the case for the corresponding leading edge regions.

361 The spatial distribution of genetic diversity can also provide valuable insights into how rear

362 edge regions have contributed to postglacial recolonizations (Cooper *et al.,* 1995; Hewitt,

363 1996, 2000; Provan & Maggs, 2012). Italian and Iberian refugial populations of *C. parallelus*

364 have not contributed to the postglacial recolonization of Europe, probably because of the

365 Pyrenees and Alps acting as significant barriers to dispersal (Cooper & Hewitt, 1993; Lunt *et*

366 *al.,* 1998). The occurrence of low diversity values in some Spanish (Sspn, PSpn) and Balkan

367 (Blg2 and Blg3) regions (Table 1 & Fig. 2) might be explained by being separated from

368 nearby populations by unsuitable habitat; therefore high genetic drift and low gene flow are

369 likely drivers of their higher genetic differentiation (Diekmann & Serrão, 2012). Anatolian

370 regions show a more complex scenario however, exhibiting the highest level of genetic

371 diversity (Table 1), and the presence of a high number of indel positions (Fig. 4 & Appendix

372 1). The occurrence of a high nucleotide and haplotype diversity, as well as high number of

373 indels, in Anatolia represents a stable rear edge that has not become genetically depauperate

374 (Cooper *et al.,* 1995; Hewitt, 1996; Dubey *et al.,* 2007; Korkmaz *et al.,* 2010). Similar

375 conclusions were also reported for several other organisms from Anatolia (Moghaddam *et al.,*

376 2000; Rokas *et al.,* 2003; Dubey *et al.,* 2007).

377 **Geographical structure of genetic differentiation**

378 It is likely that the genetic patterns observed have been greatly influenced by climatic changes

379 associated with the Pleistocene glacial periods as these are known to influence drift and gene

380 flow, which will combine particularly strongly in the postglacial recolonization phase

381 (Hewitt, 1999). For wide-ranging species, climate fluctuations may have caused range

382 contractions in some parts of the range and expansions in others (Çıplak, 2004, 2008), which

383 together may have formed the distinct genetic lineages that we know today (Hewitt, 2004;

384 Ehrich *et al.,* 2007). In addition to the possible effect of climatic changes, topographic

385 structure and mountain chains of a certain region may have led to genetic differentiation.

386 These are consistent with our findings as the analyses related with genetic structuring indicate

387 that populations of *C. parallelus* are not genetically homogenous across distribution ranges

388 (Table 2 & Figs. 3-4). The formations of the cluster A and B suggest that the Anatolian

389 Diagonal acts as an effective barrier to gene flow between the west and east regions of

390 Anatolia (Fig. 4ab). This is also supported by the presence of indel positions specific to both

391 clusters (Fig. 4ab & Appendix S1). The barrier position of Anatolian Diagonal was also

392 observed in many taxa in Anatolia (Gündüz *et al.,* 2007 and references therein). On the other

393 hand, the mainly southern distribution of the species together with some Russian individuals

394 appears to represent a relatively old genetic unit (Cluster C; Fig. 4c & Appendix S3). This

395 structuring possibly has been formed by the direct effect of glacial periods rather than the

396 geographical relationships among regions. This is further supported by presence of the most

397 common indel at site 217 in the cluster C and again could be attributed to its old age, possibly

398 resulting from a dispersal wave out of Anatolia (Fig. 4c). Moreover, this conclusion is

399 consistent with the primary distinction of some southern refugial regions from all other in

400 SAMOVA (Fig. 3) and also the component contributions (Fig. 2). At the same time, the

401 geographic position of the Cluster C may point out to a historical connection among Spain,

402 Italy, Balkans and Anatolia (Fig. 4c). Finally, the Cluster D consists of the current distribution

403 area of the species and the absence of any indel events except in some Anatolian individuals

404 suggests a relatively new dispersal wave out of Anatolia/Balkans (Fig. 4d).

405 **Phylogeography, demographic history and the barrier position of the Marmara water**

406 **body**

407 Thanks to the studies of Hewitt and his colleagues, we already have considerable knowledge

408 of the phylogeography of *C. parallelus* (Butlin *et al.,* 1991; Cooper & Hewitt, 1993; Cooper

409 *et al.,* 1995; Hewitt, 1996, 1999, 2000; Lunt *et al.,* 1998). In these studies it is assumed that

410 the present population in Europe, excluding Spain and southern Italy, is founded by a

411 population dispersing from a Balkans refugium during the last interglacial period. The

412 Spanish and Italian populations are thought to be separate units, originating from their own

413 refugial stocks, arriving to these two peninsulas in an earlier interglacial.

414 Our data is partly consistent with these findings but add more details (see Tables 1-2 &

415 Appendix S1). Our results support that the Iberia is structured into multiple groups as

416 suggested earlier (Figs. 3-4; Gomez & Lunt, 2007). A reported relationship between some

417 Spanish and Italian forms by Bella *et al.* (2007) is also accord with our findings. One of our

418 striking results is the presence of some Russian individuals within the cluster C (Fig. 4c). This

419     may be attributed to a contraction and isolation event after an old dispersal wave since there is

420     no indication of a recent expansion (Appendix S3).

421     Although a detailed discussion of the interrelationships of the populations within Anatolia is

422     out of the scope of this current study our data shows the presence of Anatolian individuals in

423     all clusters and indicates multiple expansion events from Anatolia (Fig. 4). The hypothesis of

424     gene flow between western Anatolia and the Balkans is tested by the IMA analysis, with the

425     results indicating that substantial gene flow between the Balkans and Anatolia has indeed

426     continued since the establishment of these populations. Additional mitochondrial DNA data

427     also appear to support this conclusion (Korkmaz *et al.,* 2011), and suggest that the

428     populations of SEAnt are a source of genotypes involved in this expansion. The high rate of

429     gene flow from Anatolia to Thracia indicated that Anatolia has consistently acted as an

430     important source of diversity for European regions until the Holocene (Table 3 & Fig. 6). The

431     marine barrier presented to *C. parallelus* by the Bosphorus, Dardanelles and Marmara Sea has

432     of course been influenced greatly by climate cycles. A significant decrease in sea level during

433     the last glacial maximum (Ergin *et al.,* 2007) would have led to a large part of the Marmara

434     Sea becoming a land mass across which grasshoppers may have dispersed. These results are

435     in agreement with the above suggestions that the Anatolian population contributed to the

436     establishment of central and northern European populations and in this way the genetic

437     signatures of Anatolia may have extended before the Holocene as far north as Scandinavia

438     (Fig. 4d). This conclusion is fully accord with indels distributions (Fig. 4).

439     Comprehensive data for Anatolian regions has now brought the classic model to a new stage.

440     Hewitt (1996) has previously suggested that the ultimate ancestral region is probably in

441     Anatolia, and indeed our results suggest very strongly that Anatolia is the original source of

442     European populations. We add a number of novel aspects to the classic model of *C. parallelus*

443     phylogeography including the connectedness of the Balkans and Anatolia despite the apparent

444 marine barrier. The presence of a high number of indels in Anatolia with a clinal decline from

445 Anatolia to Balkan and Europe is in support of these assumptions (Fig. 4 & Appendix 1).

446 Thus, rather than a simple, small, bottlenecked Balkans refugium we have shown that a larger,

447 more permeable Anatolian centre of origin is a much more realistic model for west

448 Palaeoarctic diversity of *C. parallelus*.

449

463

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604

605  **Supporting Information**

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

607

608  **Appendix S1** Sampling data information, accession numbers and indel variants of *cpnl-1*

609  haplotypes and shared *cpnl-1* haplotypes between regions of *Chorthippus parallelus*.

610  **Appendix S2** DAPC on R package to identify and describe all possible genetic structuring

611  across the distribution area of *C. parallelus*.

612  **Appendix S3** The results of demographic history of the clusters, IMA and the network and

613  demographic analyses of Thracian and North West Anatolian groups to investigate the barrier

614  position of the Straits;

615

616  **Biosketch**

617  Dr. E. Mahir Korkmaz an Assistant Professor at the Cumhuriyet University is interested in

618  systematics and molecular evolution of Western Palaearctic species. In particularly, he is

619  focused on Anatolian historical biogeography and mitochondrial genome evolution in insects.