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Budding speciation via peripheral isolation: the *Psorodonotus venosus* (Orthoptera, Tettigoniidae) species group example

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The peripatric or budding species model has remained theoretical until recently. The habitat preference and range pattern of the mountainous *Psorodonotus venosus* species group, distributed in Anatolia and Caucasus, offered potential to test the predictions of the model. The study aimed to confirm the parameters and to provide evidence for peripatric speciation in a terrestrial group. Fourteen populations representing the total range of the group were studied. Sequences of two mitochondrial fragments including four loci (12S rDNA+tDNAval+16S rDNA and COI) and a nuclear fragment including three loci (ITS1+5.8S rDNA+ITS2) were obtained and used in phylogenetic, time estimation, population genetics and demographic analyses. Additionally, phenotypical data were provided for the group. Phylogenetic analyses supported the monophyly of the PVG and suggested similar intralineage relationships, but did not support the monophyly of each of the species in the group. The molecular chronograms indicated radiation of the group throughout the Pleistocene. Demographic analyses suggested constant population sizes for the populations in the centre of the range but a significant departure from constancy in four peripheral populations. Genetic diversity is significantly reduced in peripheral populations, but not in others. We arrived to following conclusions. The data suggest that *P. venosus* is the ancestral species and *P. rugulosus*, *P. tendurek* and *P. hakkari* are bud species, as their haplotypes are nested within *P. venosus*. As expected under the budding speciation model, (i) in contrast to ancestral species, genetic diversity is reduced, and there are signs of departure from constant population size in bud species; (ii) bud species have isolated and restricted ranges, while the ancestral species does not, (iii) the relative ages of ancestral and bud species are the most reliable data to confirm the model, and chronograms of PVG well support this prediction, and (iv) the divergence events in the group co-occurred with major climatic transformations of the Pleistocene.

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Introduction

Understanding the general patterns and processes of speciation is fundamental in explaining the diversity of life. An enormous amount of research has been performed on speciation since Darwin and different modes of speciation have been defined. Ecologists and population geneticists mostly follow the categorization based on spatial criteria suggested by Mayr in 1942 (Mayr & Ashlock 1992) as allopatric, sympatric, peripatric and parapatric (Losos & Glor 2003). Some other authorities suggest that spatial contexts should not be used as a dominant criterion for classifying speciation processes (Butlin *et al.* 2008, 2012; Fitzpatrick *et al.* 2009). Butlin *et al.* (2008, 2012) suggest that every speciation event is unique, and the classification should consider the underlying evolutionary forces, ecological circumstances and genetic mechanisms. On the other hand, taxonomists and phylogeneticists tend to use phylogenetic approaches and define modes of speciation in terms of

relationships of the speciation's products (Hennig 1966; Hörandl & Stuessy 2010; Podani 2013): (i) the splitting of an ancestral species into two daughter species as cladogenetic speciation, (ii) the transformation of the ancestral species into a new one as anagenetic speciation, (iii) the merging of two ancestral populations as hybrid speciation and (iv) the divergence of a peripheral isolate while the ancestor remains extant as budding speciation. However, budding speciation has been equated to anagenetic speciation in some publications (Stuessy *et al.* 2006; Patino *et al.* 2014). The spatial and phylogenetic classifications have been rarely related to each other. The most widely related modes are budding and peripatric speciation. Progress in molecular biology and bioinformatics in the last two–three decades has led to a revolution in the study of speciation.

Budding speciation assumes rapid divergence of peripheral isolates due to founder effects, genetic drift and new ecological conditions (Frey 1993; Coyne & Orr 2004; Stuessy *et al.* 2006; Losos & Ricklefs 2009; Tedesco *et al.* 2012; Patino *et al.* 2014). Frey (1993) defined three main reasons causing the formation of peripheral isolates: waif dispersal, microvicariance and range retraction. Although the budding speciation model was considered the predominant model of speciation (Mayr & Ashlock 1992; Frey 1993), a Web of Science search finds very few studies conducted on natural populations/lineages. Peripheral isolation, in general, and microvicariance and range retraction, specifically, have remained theoretical until very recently (Coyne & Orr 2004), especially for non-island biotas, and there have been only a few empirical studies on continental lineages (Gray *et al.* 2008; Kim *et al.* 2013; Kruckenhauser *et al.* 2013; Takayama *et al.* 2013; Toussaint *et al.* 2013; Blair *et al.* 2014). Limited number of studies may be because of fuzzy parameters of the model or their overlap with those of others.

The geographical sizes of ancestral populations and peripheral isolates (plesiomorphic and apomorphic populations, respectively, *sensu* Frey 1993) were the main parameters on which the model was based (Mayr & Ashlock 1992). This was a reason why the model was widely used to explain island endemicity, where speciation starts with an initial asymmetry in population size (Frey 1993; Losos & Glor 2003; Losos & Ricklefs 2009). However, the inequality in range size is more properly modelled as a fuzzy parameter, as a numerical threshold is not logical. Thus, inequality in range size is informative when used in combination with other parameters. Second, because the peripheral populations are assumed to be small, the bud species must show signs of founder effects or genetic drift (Coyne & Orr 2004; Stuessy *et al.* 2006; Losos & Ricklefs 2009; Tedesco *et al.* 2012; Kim *et al.* 2013; Patino *et al.* 2014). However, such signs are also possible when a non-bud species experienced a historical bottleneck. The third and the most reliable is the paraphyly of species (Funk & Omland 2003; Hörandl & Stuessy 2010), and that may be uncertain when a species tree is used, but more reliable when a gene tree is used. Thus, this parameter will not be very reliable without a gene tree produced from a sufficient number of samples (Frey 1993). Paraphyly is also a case for young species (Funk & Omland 2003). The age of the ancestral species seems to be the most credible basis for identifying a budding speciation, as the ancestor should remain extant prior, during and after the divergence of the peripheral isolates.

The rear/leading edge model defines the pattern and consequences of range changes of cold-adapted populations, especially due to the glacial cycles of the Pleistocene (Hewitt 1996; Çıplak 2004; Hampe & Petit 2005). The model assumes that the range of a population expands on its front edge while eroding from the opposite rear edge. During an interglacial, populations' ranges might expand from their northern-leading edge while eroding from the southern-rear edge. Depending on the altitudinal heterogeneity, several small patch populations may remain on highlands located along the eroding edge of the populations. Persistence of these small populations for sufficient time to diverge to new species might have resulted in budding speciation (Frey 1993). The Anatolio-Caucasian glacial refugial system constitutes a natural laboratory to study budding speciation due to peripheral isolation because it has several summits higher than 2000 m. The importance of the area and other glacial refugia of the western Palaearctic has not been well studied in this respect.

The genus *Psorodonotus* (Orthoptera, Tettigoniidae) ranges over Anatolia, Caucasus and the Balkans. It comprises 15 species (Eades *et al.* 2015), and three species groups were suggested on the basis of their morphologies: the *P. specularis*, *P. caucasicus* and *P. venosus* groups (Kaya *et al.* 2013a, 2014, 2015a). They occur in moist meadow habitats at elevations above 1800 m. The *P. venosus* group (PVG hereafter) includes four named species: *P. venosus*, *P. rugulosus*, *P. hakkari* and *P. tendurek* (Kaya *et al.* 2013a). Its distribution range is bordered by the Greater Caucasus in the north and extends to the edge of the Zagros Mountains in the south along the eastern side of Turkey. Species other than *P. venosus* occur as patchy populations in the southern or western parts of their ranges. By its habitat preference and range pattern, the PVG constitutes a candidate model group for the study of peripheral isolation and budding speciation resulting from Pleistocene climatic cycles in the Anatolio-Caucasian refugium.

The present study aimed to investigate speciation and the phylogeography of the *P. venosus* group, in particular, to test the possibility of budding speciation due to range

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fragmentation caused by climatic oscillations in the Pleistocene. The model criteria are considered as the hypotheses to be proven or disproven by the data obtained from four mitochondrial and three nuclear markers. The following results will be considered as evidence for budding speciation via range retraction caused by climatic oscillations in the Pleistocene: (i) paraphyly of haplotypes, or the nesting of some species within others, (ii) reduced genetic diversity or signs of founder effects/genetic drift in the history of peripheral or bud species, but not in populations of putative ancestral species, (iii) older ancestral species and younger peripheral species, (iv) restricted range of the peripheral species and (v) divergence/speciation events correlating with the climatic oscillations of the Pleistocene.

Apart from studying the speciation pattern of the group, data may enable the estimation of the substitution rate for the genetic markers used. The phylogeny and range pattern of *Psorodonotus* (Kaya *et al.* 2013a, 2014, 2015a; unpublished data) indicate that there is a correlation between the radiation of the genus and the geological evolution of Çoruh Valley in north-east Turkey. This valley was hollowed out rapidly 6–5 Myr by the development of the North Anatolian Fault Zone (Ketin 1976; see also Sosson *et al.* 2010). Perhaps the *P. specularis* + PVG clade originated from an ancestral stock that remained in the east side of this deep valley, while all others arose from a stock that remained on the western side. Thus, time estimation analyses that are calibrated either by presently introduced rates or by the age of the valley can be used to corroborate each other and to estimate divergence rates for each marker.

Materials and methods

Study area, sampling and taxonomy

The high plateau located between Turkey, Armenia and Georgia, which originates from different tectonic plates (Sosson *et al.* 2010), constitutes the main range distribution area of the PVG. Including distant and disjoint representatives, this area extends to the Greater Caucasus in the north and to the northern end of Zagros Mountains in the south, to the highland covered by the Aras River and Urumiah basins in the east and to the western end of Pontic Taurus in the west. Members of the PVG live in alpine moist meadows over 1800 m in elevation. The range area is considered to be a glacial refugial system (Tarkhnishvili *et al.* 2000; Seddon *et al.* 2002; Sağlam *et al.* 2013), and the summits there considered to be intrarefugial arctic poles during glacials and as sky-islands during interglacials for the mountainous populations (Çıplak 2004; 2008; Çıplak *et al.* 2015) because the peaks over 1800 m were glaciated during cold periods (Sarıkaya *et al.* 2011). The habitat preference of the PVG and the topography of the range area make the lineage a candidate model group for the study of the possible effects of glacial cycles on speciation.

Fourteen populations representing the entire range and taxonomic diversity of the PVG were sampled (Table 1; Fig. 1). To estimate relationships of the PVG with other members of the genus *Psorodonotus*, representatives from other lineages were included in analyses. The present taxonomy of *Psorodonotus* is phenotype based (upon morphology and male calling song; Kaya *et al.* 2013a, 2014, 2015a). According to these studies, the PVG includes four species: *P. venosus*, *P. rugulosus*, *P. tendurek* and *P. hakkari*; the last three are monotypic, while the first is polytypic (Stolyarov 1983). Although these subspecies cannot be easily distinguished, their type localities from Georgia and Armenia were sampled. Morphologic and bioacoustic analyses given in Kaya *et al.* (2013a) are repeated by adding new specimens to obtain a more robust group-level taxonomy (Appendix S4 & 5). We assumed it unnecessary to apply species delimitation tests to the genetic data, as reciprocal monophyly is a preliminary requirement for the present approaches (Knowles & Carstens 2007; Carstens *et al.* 2013), contrary to our working hypotheses.

Laboratory procedures and data preparation

Mitochondrial markers are more advantageous than nuclear markers at elucidating recent evolutionary events (Avise 2000). Nuclear markers may be more informative at detecting gene flow between populations, but they contain low variation for species-level studies in Orthoptera (Fenn *et al.* 2008; Çıplak *et al.* 2015). The sequences of two mitochondrial fragments including four loci (12S rDNA+tDNA-val+16S rDNA and cytochrome C oxidase subunit I) and a nuclear fragment including three loci (internal transcribed spacers 1 and 2 and 5.8S rDNA in between) were obtained and used in phylogenetic, time estimation, population genetics and demographic analyses. DNA extraction and PCR amplification procedures are given in Appendix S1 (Çıplak *et al.* 2010; Kaya *et al.* 2013b). The nucleotide sequences of each unique haplotype identified were deposited in the GenBank database (see Table S1). Sixteen COI sequences of *Psorodonotus* were obtained from GenBank (Kaya *et al.* 2015a).

Genetic structure and historical demography of populations

Genetic structure and historical demography of the populations of the PVG were estimated using COI sequences, the markers for which we have sufficient samples. Diversity parameters such as the number of unique haplotypes (k), haplotype diversity (h), number of segregating sites (s), nucleotide diversity (π) and mean number of pairwise differences between the k haplotypes (d) were estimated using ARLEQUIN v.3.5.1.2 (Excoffier & Lischer 2010).

Table 1 Sampling details (see Fig. 1) of the *P. venosus* group specimens

Species	Population	Abb.	Coordinates	Elevation (m)	Date
rugulosus	Turkey, Giresun, Avşar Yayla	TGA	40° 27.961N, 038°42.487E	2260	30.07.2012
	Turkey, Erzurum, Yayla Pass	TEY	40°26.656°N, 041°37.480 E	2284	03.08.2012
tendurek	Turkey, Van, Tendürek Pass	TVT	39° 23.20.9N, 043° 59.066E	2412	17.07.2011
hakkari	Turkey, Hakkari, Yüksekova	THY	37°51.699°N, 44°38.215E	1902	11.07.2009
venosus	Turkey, Ağrı, Taşlıçay	TAT	39°43.525N, 43°28.893E	2302–2365	16.07.2011
	Turkey, Kars, Kağızman	TKK	40°03.040N, 43°19.677E	2338	05.08.2012
	Turkey, Ardahan, Posof	TAP	41°25.068N, 42°44.564E	2540	04.08.2012
	Turkey, Ardahan, Çıldır	TAC	41°07.476N, 042°54.38.2E	1940	04.08.2012
	Turkey, Ardahan, Yalnızçam	TAY	41°03.882N, 42° 28.139E	1840–1930	28.07.2010
	Turkey, Artvin, Çam Pass	TAA	41°12.154N, 042° 30.390E	2640	15.07.2011
	Turkey, Ardahan, Göle	TAG	40°45.700N, 042°34.253E	2106	03.08.2012
	Georgia, Lesser Caucasus, Tskhratskharo Pass	GLC	41°38.882N, 43°29.948E	2310	07.08.2014
	Georgia, Greater Caucasus, Jvari Pass	GGC	42°30.820N, 44°27.689E	2370	08.08.2014
	Georgia, Greater Caucasus, Shatili Pass		42°31.338N, 45°3.398E	2700	12.08.2014
	Armenia, Syunik, Mehri Pass	ASM	39°6.314N, 46°8.045E	2900	03.08.2014

As founder effects and genetic drift are expected for the peripherally isolated populations (Frey 1993), present genetic structure and demographic history may contain crucial signs indicative of budding speciation. Differentiation between the populations of the PVG was estimated using pairwise gamma$_{ST}$ statistics using DnaSP v.5.10.1 (Rozas 2009) as this parameter is more appropriate for mtDNA sequence data and corrects for errors associated with incomplete sampling of populations (Hudson *et al.* 1992). Past population expansions and bottlenecks were estimated by performing Tajima's *D* (Tajima 1989), Fu's F_S (Fu 1997), Fu and Li's *F** (Fu & Li 1993) and Ramos-Onsins & Rozas' R_2 (Ramos-Onsins & Rozas' 2002) tests for mutation–drift equilibrium. A coalescence-based approach was used to estimate the exponential growth rate (*g*) of the populations. The demographic history of each population was also explored by mismatch distribution analysis of observed haplotype pairwise differences following a sudden population expansion model (Rogers & Harpending 1992). The parameters of the demographic expansion τ, θ_0 and θ_1 were estimated by a generalized nonlinear least-squares approach. To estimate the significance of the Harpending's raggedness index (Hri; Harpending 1994) and the sum of squared deviations (SSD) between the observed and expected mismatch distributions, a parametric bootstrapping approach (Schneider & Excoffier 1999) was used with 1000 replicates. Historical dynamics of populations that show signs of departure from constant population size were also investigated using a coalescence approach. The GMRF Bayesian skyride plots (Drummond *et al.* 2005; Minin *et al.* 2008) model generates a posterior distribution of effective population size through time using Markov chain Monte Carlo (MCMC) sampling (Appendix S1).

Phylogenetic analyses

Paraphyletic gene trees are more informative than species trees at determining the paraphyly of ancestral/bud species (Funk & Omland 2003). Thus, robust haplotype trees have special importance to test the core hypotheses of the study. Four data matrices were produced: (i) the COI, (ii) the 12S rDNA+tDNAval+16S rDNA (12S–16S hereafter), (iii) concatenated COI+12S–16S and (iv) the ITS1+5.8S rDNA+ITS2 (ITS hereafter). MEGA v.6 (Tamura *et al.* 2013) was used to calculate the nucleotide composition, the number of variable and parsimony-informative sites and the ratio of transition to transversion. The first three matrices were analysed separately under maximum parsimony (MP), maximum-likelihood (ML) and Bayesian inference (BI) approaches (for details, see SI-1), but the results of concatenated matrix are presented in the main text and those of COI and 12S–16S are presented in Appendix S3. To determine monophyly within the PVG and its relationship with other members of the genus, we included representative haplotypes from other species of *Psorodonotus*.

Estimation of divergence times

The relative age of the haplotypes and the populations/species comprising them is crucial for the study, as an older ancestral species and younger peripheral species would be considered the most convincing evidence of budding speciation. Additionally, robust time estimation is essential to determine whether the PVG radiated in the Pleistocene or not. The ITS matrix was not subjected to time estimation analysis because there was too little variation present. The COI and 12S-16S matrices were separately analysed using BEAST v.1.8.0 (Drummond *et al.* 2012). Diversification rate shifts for sequences of both matrices were tested using the topological programme SymmeTREE v.1.1 (Chan &

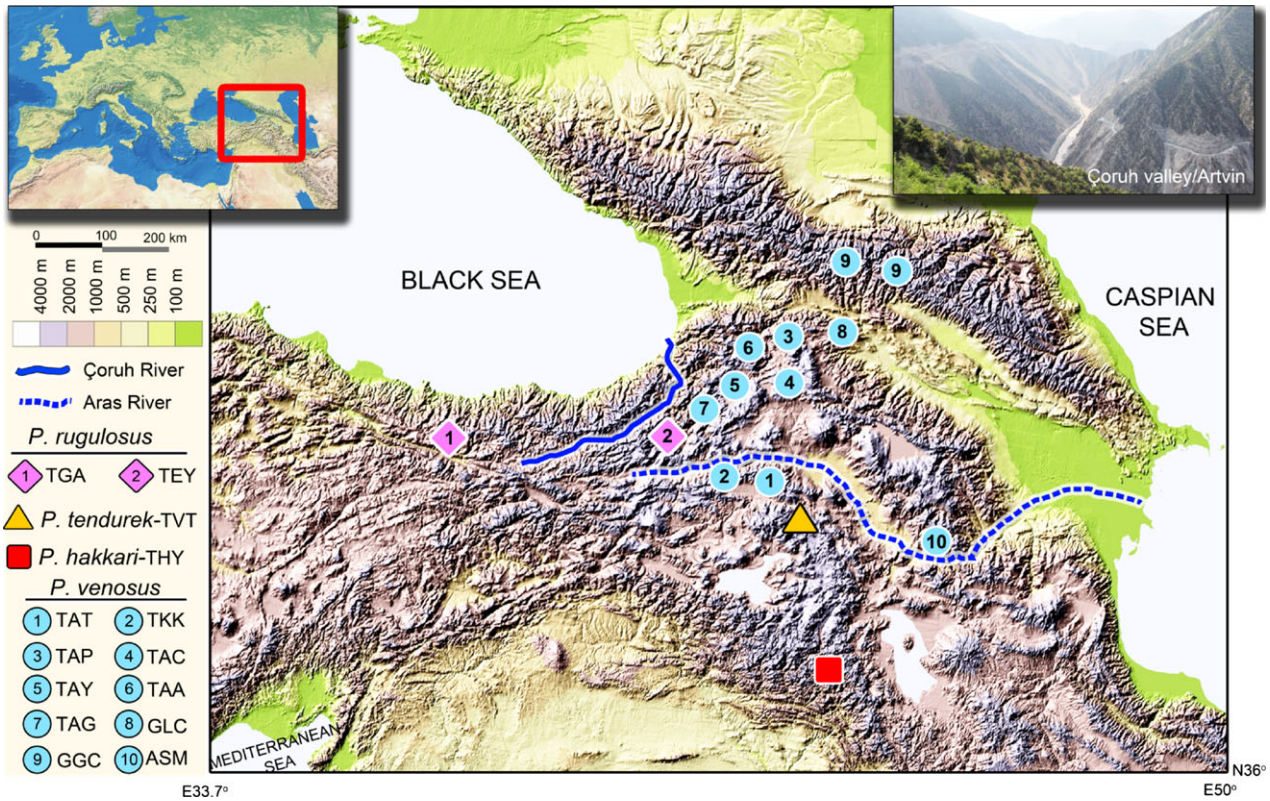

Fig. 1 Sampling localities of the PVG (see Table 1 for details).

Moore 2005) (Appendix S3). The nodes of the main clades suggested by the each matrix were dated using a Bayesian coalescence approach, as implemented in BEAST. The time to the most recent common ancestor (TMRCA) for each clade was estimated under the model parameters highlighted in jMODELTEST v.2 (Darriba *et al.* 2012) (Appendix S1). We followed two strategies to calibrate the time estimation analyses. First, we benefitted from the previously introduced divergence rates for insects. Although different rates have previously been reported for COI, the figure of 0.017 substitutions/per site/per Myr (subs/s/Myr) is the most common rate for insects (Papadopoulou *et al.* 2010) and Orthoptera (Allegrucci *et al.* 2011), and the BEAST analysis of the COI matrix was calibrated accordingly. Because there is no estimation of the mutation rate for the rRNA genes of insect mitogenomes, the BEAST analysis of the 12S-16S matrix was run as 'linked' jointly with the COI matrix. Second, we conducted BEAST analyses for each data matrix setting the divergence of *P. specularis* + PVG from the others to 5.5 Myr (±0.2), the age of Çoruh Valley. This allowed us to test previously reported divergence rates for COI and to estimate a divergence rate for the 12S rDNA+tDNAval+16S rDNA segment. Each BEAST analysis was repeated under strict and relaxed clock options. The Bayes factor (BF) was used to evaluate the robustness of estimations, and the highest BF was used to draw conclusions.

Results

Sequence attributes and descriptive genetics

Sequences attributes and the model parameters for each of the COI, 12S–16S, COI+12S–16S and ITS matrices are given in Table 2. The number of unique haplotypes per gene and per population is given in Table S2. *P. venosus* and *P. rugulosus* are the only two species sharing one haplotype per COI and 12S–16S. Other haplotypes of both markers are unique to species and mostly to the sampled population. No intraindividual sequence variation was detected in the ITS, and a total of five haplotypes were found in the PVG. The TAP-T1 is shared by all of the populations of the PVG except THY, while the others are unique to the sampled populations (Table S2). Genetic diversity indices were calculated for nine populations, but not for the other five, as two of those five (THY-*P. hakkari* and TVT-*P. tendurek*) contain a single haplotype, and the sample size is <5 in the remaining three (TEY, TAG and ASM) (Table 3). The lowest haplotype and nucleotide diversity indices were recorded in two populations of

Table 2 Description of the sequences and matrices used in the study

Sequence attributes		COI	12S–16S	COI+12S–16S	ITS
No. of PVG sequences		218	124		143
Final length after alignment and trimming		1098	705	1941	845
No. of constant sites		740	481	1408	840
No. of variable sites		358	224	533	5
No. of indel sites			21		
No. of parsimony-informative sites		297	148	477	5
No. of unique haplotypes without (with) indels for PVG		71	22 (23)		
No. of non-PVG ingroup haplotypes	*P. davisi*	3	3	3	
	P. specularis	3	3	3	
	P. soganli	2	3	3	
	P. fieberi	6	2	6	
	P. illyricus	3			
	P. macedonicus	5			
	P. caucasicus	3	3	3	
Outgroups	*Pholidoptera* sp.	1	1	2	
	Parapholidoptera sp.		1		
	Eupholidoptera sp.		1		
	Bolua sp.	1			
No. of unique haplotypes without indels in the matrix		98	42	76	5
Model parameters suggested by JMODELTEST		TVM+I (0.616) + G (1.1316)	HKY+G (0.253)	TVM+I (0.618) + G (1.349)	

P. rugulosus (TGA and TEY) and a peripheral population of *P. venosus* (GGC), while the highest haplotype and nucleotide diversity values as well as segregating sites were observed in the populations of *P. venosus* located around the Ardahan Plateau (TAP, TAC, TAY, GLC and TAG).

Genetic structure and historical demography of populations

Gamma$_{ST}$ values between population pairs belonging to the PVG are in support of morphospecies. The gamma$_{ST}$ values of pairs of populations located in the periphery of the PVG's range are >0.90 (Table 4). These populations diverged from the remaining populations of the group with a 0.6 gamma$_{ST}$ value on average except for the pairs formed with TAP and TEY-TAG. The gamma$_{ST}$ between the Armenian population of *P. venosus* (ASM) and each of the TEY, TGA, THY and TVT ranges from 0.941 to 1.00, but the sample size of ASM is small. The most divergent three populations of *P. venosus* are GGC, TAT and TKK. The low gamma$_{ST}$ values between pairs formed with the TAY, TAP, TAC, TAA and GLC populations and that between TEY and TAG indicate low level of divergence or gene flow.

To visualize divergence between populations, a principal coordinate analysis (PCoA) was performed using the gamma$_{ST}$ values (Fig. 2). THY and TVT separated from all the others along coordinate 1. The TEY and TGA populations of *P. rugulosus* differ from the others in coordinate 1. and from each other in coordinate 2. Both the clusters TAT+TKK and ASM separated from the remaining populations of *P. venosus*, but all show affinities with other populations of the species. The remaining seven populations of *P. venosus* from Ardahan Plateau constitute a cluster. The clusters obtained from gamma$_{ST}$ and phenetic data are well congruent. The genetic and phenetic clustering patterns are inconsistent for the following populations: (i) the TEY population of *P. rugulosus* is similar to other populations of the species (TGA) phenetically, but intermediate to *P. rugulosus* and *P. venosus* genetically, (ii) the TAT+TKK and ASM populations are genetically divergent from other populations of *P. venosus*, but are similar phenetically (compare Fig. 2 and Figs S4&5). In summary, the TEY, TGA (*P. rugulosus*), THY (*P. hakkari*) and TVT (*P. tendurek*) populations are both phenotypically and genotypically distinct, while *P. venosus* is distinct in phenotype, but is genetically heterogeneous.

Demographic tests were not applied to the TEY, GLC and ASM populations, due to their small sample sizes (<10). TVT and THY populations were also excluded from the analysis because each contained a single haplotype. Five of the remaining eight populations suggested no statistically significant departure from constant population size (Table 5). The Fu's F_S, g parameter and the skyride plot suggest a contraction for TAT approximately 100 kyr ago. All demographic tests suggested an expansion for TGA (*P. rugulosus*) approximately 50 kyr ago during the last glacial period (Fig. 3). The low haplotype diversity and the low number of segregating sites in TGA are consistent with the demographic results.

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Table 3 Genetic diversity indices of 14 populations of the PVG (shown from left to right are sample sizes (N), the number of haplotypes without indels (*k*), the number of segregating sites (*s*), haplotype diversity (*h*), nucleotide diversity (π), mean number of pairwise differences between the *k* haplotypes (*d*)). The associated standard deviations (SD) are given just after the value. Indices were calculated using the model HKY+G (0.0150)

Species	Population	N	*k*	*s*	*h* ± SD	π ± SD	*d* ± SD
rugulosus	TEY	4	3	8	NA	0.003813 ± 0.002850	4.186940 ± 2.620620
	TGA	33	8	7	0.3845 ± 0.1082	0.000391 ±0.000403	0.429017 ± 0.397606
hakkari	THY	13	1	NA	NA	NA	NA
tendurek	TVT	21	1	NA	NA	NA	NA
venosus	TAT	20	5	27	0.7579 ± 0.0575	0.009263 ± 0.004933	10.170481 ± 4.849443
	TKK	15	5	22	0.7810 ± 0.0644	0.005720 ± 0.003225	6.280549 ± 3.157776
	TAG	2	2	44	NA	0.054485 ± 0.054939	59.824660 ± 42.654511
	TAY	10	6	57	0.8444 ± 0.1029	0.019707 ± 0.010755	21.637808 ± 10.441623
	TAP	28	20	79	0.9735 ± 0.0162	0.023645 ± 0.011902	25.962404 ± 11.737485
	TAA	27	13	62	0.8746 ± 0.0517	0.014363 ± 0.007366	15.770213 ± 7.262426
	TAC	18	10	43	0.9150 ± 0.0405	0.016448 ± 0.008565	18.059444 ± 8.409662
	GGC	17	7	22	0.6544 ± 0.1223	0.008572 ± 0.004634	9.411765 ± 4.546751
	GLC	8	7	40	0.9643 ± 0.0772	0.018508 ± 0.010448	20.321429 ± 10.071115
	ASM	2	1	NA	NA	NA	NA

Table 4 Pairwise gamma$_{ST}$ values for 14 populations belonging to four species of PVG

	rugulosus		*hakkari*	*tendurek*	*venosus*								
	TEY	TGA	THY	TVT	TAT	TKK	TAG	TAY	TAP	TAC	TAA	GGC	GLC
TGA	0.763												
THY	0.945	0.979											
TVT	0.946	0.984	1.000										
TAT	0.380	0.684	0.704	0.749									
TKK	0.586	0.812	0.843	0.870	0.152								
TAG	0.368	0.519	0.634	0.612	0.196	0.332							
TAY	0.430	0.676	0.603	0.576	0.428	0.509	0.175						
TAP	0.217	0.542	0.373	0.361	0.357	0.371	0.051	0.042					
TAC	0.334	0.684	0.552	0.543	0.477	0.501	0.134	0.040	0.075				
TAA	0.383	0.686	0.554	0.536	0.472	0.519	0.145	0.024	0.039	0.038			
GGC	0.570	0.809	0.680	0.698	0.570	0.644	0.159	0.387	0.191	0.424	0.377		
GLC	0.523	0.714	0.623	0.583	0.458	0.556	0.158	0.216	0.047	0.192	0.161	0.161	
ASM	0.907	0.923	1.000	1.000	0.450	0.645	0.630	0.440	0.189	0.300	0.344	0.506	0.499

Phylogeny

ITS sequences of the PVG contain fewer variable positions, while the mtDNA sequences exhibited a significant variation (Table 2). Prior to phylogenetic analyses, we tested for range shift in the COI and 12S–16S sequences using SymmeTREE (Appendix S3). The SymmeTREE suggested range shifts for the generic and PVG nodes. Congruent with the range shift patterns, inconsistency between the trees occurs mainly in the generic and the PVG nodes. Trees produced from each of the COI and 12S–16S matrices are presented in Fig. S3, and those from the concatenated matrix plus ITS haplotype network in Fig. 4.

The maximum parsimony (MP) analysis applied to the concatenated matrix resulted in 539 equally parsimonious trees (tree length = 1118, CI = 0.582, RI = 0.897, RC = 0.522). The analyses using BI, ML and MP all resulted in similar topologies (Fig. 4A). Monophyly of the genus and of the PVG received high bootstrap/posterior probability supports, but these trees did not resolve the polytomy at the base of the genus (Fig. 4A). The branch containing the single haplotype of ASM splits off in the base of PVG from all the others. In the next step, there are two phylogroups. PhG1 includes two subphylogroups: PhG1A includes all haplotypes of TGA+TEY (*P. rugulosus*) plus representative haplotypes from TAG, TAY, TAP and

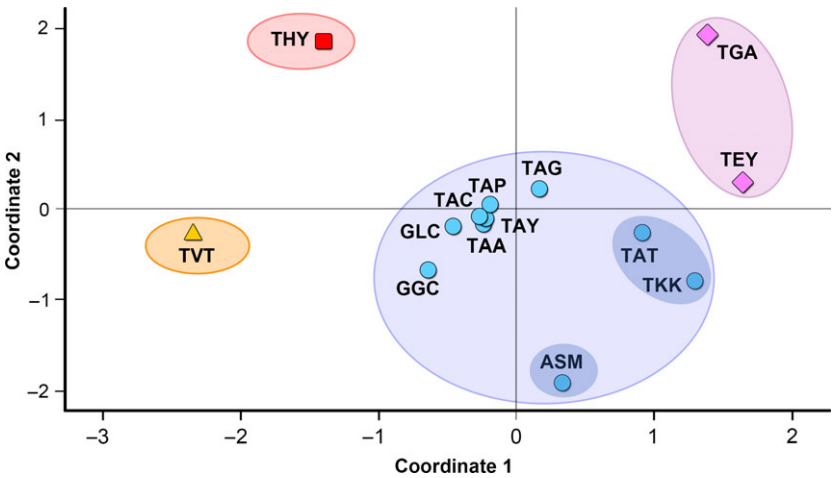

Fig. 2 Principal coordinate analysis of pairwise gamma$_{ST}$ between the 14 populations of PVG (given in Table 4).

Table 5 Historical demographic estimates for 14 populations of PVG

Species	Population	N	*k*	Tajima's *D*	Fu's *Fs*	Fu and Li's *F**	R_2 statistics	growth rate *g*	SSD	Hri	Tau	θ_0	θ_1
rugulosus	TEY	4	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	TGA	33	8	−2.179**	−8.080***	−3.874**	0.062*	15754.49	0.221	0.207	2.961	0.000	3.6
hakkari	THY	13	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
tendurek	TVT	21	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
venosus	TAT	20	5	0.869	7.661	−1.327	0.133	−249.78	0.138**	0.125*	17.180	0.000	21.359
	TKK	15	5	−0.676	3.438	0.553	0.124	−173.18	0.0290	0.061	0.508	1.593	23.931
	TAY	10	10	−0.566	3.893	−0.445	0.147	−39.95	0.0762	0.069	33.227	0.002	5.687
	TAP	28	20	0.272	-0.814	−0.576	0.134	32.78	0.026*	0.010	37.084	0.002	58.379
	TAC	27	13	−0.641	1.852	−1.039	0.111	−58.18	0.045	0.042	0.285	3.614	1.000
	TAA	18	10	0.964	2.505	1.234	0.174	−43.83	0.041	0.031	34.852	0.002	11.928
	TAG	2	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	GGC	17	7	1.780	4.921	1.332	0.190	−137.30	0.124	0.115	18.965	0.000	1.740
	GGL	8	7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	ASM	2	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

TAC (*P. venosus*), and PhG1B includes six haplotypes of TAT+TKK. PhG2 also contains two subphylogroups: PhG2A includes haplotypes from the TVT (*P. tendurek*), THY (*P. hakkari*), GLC, GGC, TAY, TAP, TAA and TAC populations (*P. venosus*), while PhG2B contains haplotypes from the remaining six populations. The single haplotype of *P. hakkari* and *P. tendurek* occurs as a sister group that branches off at the base of PhG2B.

Estimation of divergence time

Low levels of variation in ITS sequences prevented us from using them in time estimation. As SymmeTREE analyses suggested the existence of branch-dependent variation in diversification rates for each of COI and 12S–16S (see Appendix S3), we applied different analyses to each of these matrices to use them to anchor each other. The nodes that received strong supports in the tree topology were used to constrain the divergence time analyses (Appendix S3). BEAST analyses of each matrix were run under the model parameters given in Table 2. The mean TMRCAs, the HPD and the BF for each analysis are given in Table 6, and the means are also indicated in Figs 5 and 6.

Of the three BEAST runs for 12S-16S that calibrated by linking to COI suggested considerably different TMRCs for the main nodes than the remaining six runs, which produced similar results. However, the BEAST runs under relaxed clock model for Çoruh River-calibrated COI and 12S-16S as well as the rate-calibrated COI have higher BF values. BEAST results combining these three analyses estimated a date for the TMRCA of *Psorodonotus* at 5.73–6.34 Myr, corresponding to the end of the Messinian and the development of Çoruh Valley. The *P. specularis*+PVG

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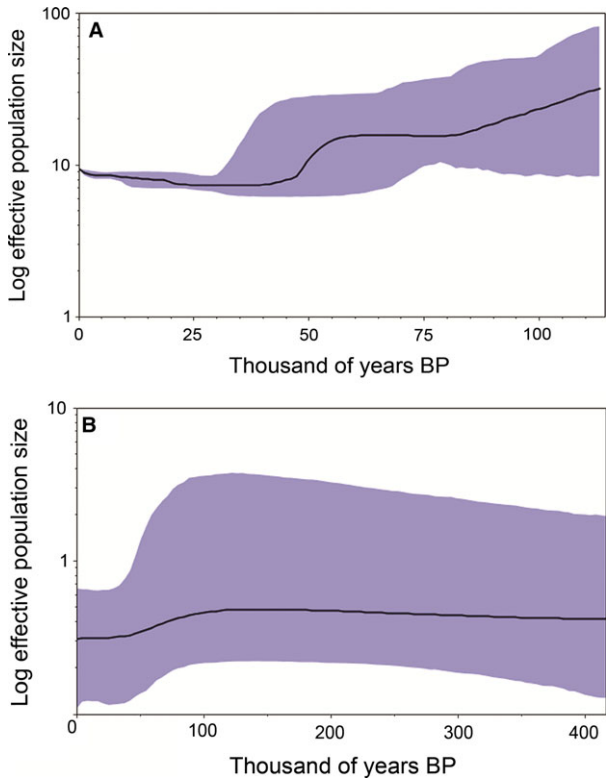

Fig. 3 Demographic history of the TGA and TAT populations as estimated with GMRF Bayesian skyride plots. Thick line shows the median predicted population size (N_e x τ; τ = generation length in units of time) on logarithmic scale; the shaded area represents the 95% highest posterior density (HPD), and lines indicate median estimates of the time to the most recent common ancestor.

lineage ranging in the east of Çoruh shared an MRCA as late as 4.11-4.56 Myr in the Early Pliocene following the development of Çoruh Valley. The TMRCA for the PVG is dated at 2.05–2.94 Myr at the Pliocene–Pleistocene transition. Within PVG, the TMRCAs for each phylogroup are dated at 1.29-1.80 Myr for PhG1 and 1.32-1.80 Mya for PhG2. *P. rugulosus* budded off from PhG1 at 1.00–1.21 Myr and *P. hakkari* + *P. tendurek* from PhG2 1.06-1.47 Myr during the Mid-Pleistocene transition (MPT) when glacial periods extended from 41 to 100 Kyr. *P. hakkari* and *P. tendurek* diverged from each other prior to the four intense glaciation periods approximately 0.55–0.94 Myr.

Molecular analyses were calibrated by the geological development of Çoruh Valley also allowed us to estimate a substitution rate for the markers used. BF of the BEAST analyses under the relaxed clock model for both COI and 12S–16S was higher than that obtained under the strict clock model (Table 7). In general, the substitution rates inferred from BEAST analyses largely confirm each other, giving similar TMRCA. The mean substitution rates for COI are slightly higher than the commonly imputed rate (0.0177 subs/s/Myr) and are very similar under relaxed (0.018197 ± 9.438^{-5} subs/s/Myr) and strict (0.018664 ± 1.746^{-5} subs/s/Myr) clock models. River-calibrated BEAST runs for the 12S–16S fragment produced similar TMRCAs for the main nodes in *Psorodonotus*, indicating substitution rates of 0.009732 ± 8.028^{-6} (strict) and 0.011884 ± 3.863^{-6} (relaxed) subs/s/Myr.

Discussion

Phylogenetic analyses have well supported the monophyly of both *Psorodonotus* and the PVG, but not that of the four phenetic species in the group (Fig. 4; Appendix S3). Of the four species, *P. tendurek* and *P. hakkari* occur as subclades of *P. venosus*. TGA and TEY are two populations representing *P. rugulosus*. Haplotypes of TGA constitute an independent phylogroup for each marker but still lie within *P. venosus*. In contrast, TEY is a complicated case, as it shares no mt-haplotype with TGA; instead, it shares mt-haplotypes with TAP (*P. venosus*) in addition to a few population-specific haplotypes. Interestingly, the common and population-specific haplotypes of TEY together with a few haplotypes from *P. venosus* constitute a sister phylogroup to the group formed by the haplotypes of TGA. Although TEY+TGA constitute a distinct phenetic unit, indeed the most aberrant unit of the PVG (Appendix S4&S5; Kaya *et al.* 2013a), that unit is genetically related to populations of *P. venosus*. Clear morphological and bioacoustic similarities of TGA and TEY can be interpreted as a reflection of the overall similarity of their nuclear genomes as for their ITS sequences. All of these evidences together indicate that *P. venosus* is paraphyletic, and the other three species constitute phylogroups internal to it. This conclusion is well in accord with the suggestion that the bud species rendered ancestral species paraphyletic (Frey 1993; Funk & Omland 2003; Losos & Glor 2003; Losos & Ricklefs 2009). *P. rugulosus* is also paraphyletic, as haplotypes found in TEY constitute an independent phylogroup within the *P. venosus* haplotypes (Fig. 4).

The phenetic clusters contradict the phylogenetic pattern but largely fit to clusters produced by $gamma_{ST}$ (Fig. 2). Of the ten populations of *P. venosus*, seven constitute a single cluster both genetically and phenetically. The remaining TAT, TKK and ASM populations are genetically divergent but still show affinities with the other seven populations. The TVT (*P. tendurek*), THY (*P. hakkari*) and TGA (*P. rugulosus*) populations are genetically divergent from all the others. The TEY population belongs to *P. rugulosus*, but is genetically divergent from TGA. These results support the independence of their gene pool. Thus, *P. venosus* seems to be the ancestral species, and the other

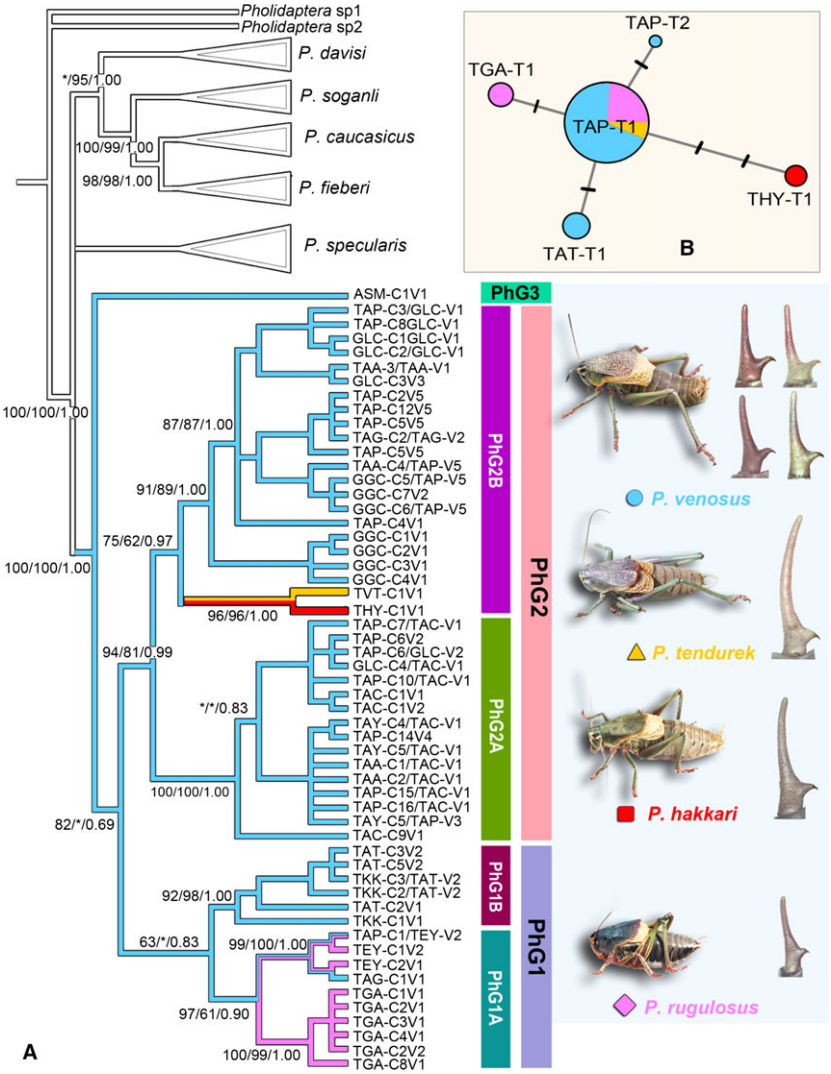

Fig. 4 A- Phylogenetic tree produced using the COI+12S-16S data set. The numbers on the nodes show bootstrap (MP and ML) or posterior probability (BI) values for MP, ML and BI, respectively. * indicates a non-supported node in the respective analysis. Haplotype symbols established by the abbreviations of concatenated haplotypes of COI and 12S-16S (see Table S2). B- A median-joining network illustrating the relationships among the five ITS1+5.8S+ITS2 haplotypes of the PVG scored in 143 individuals.

three are derived bud species. These conclusions give rise to two questions to be answered. First is the case of TEY (*P. rugulosus*). Pairwise gamma$_{ST}$ values between TEY and TVT, THY and its con-specific TGA are >0.9 but <0.8 between pairs formed with populations of *P. venosus* and are even 0.368 with parapatric TAG. However, the ITS haplotype TAY-T1 is shared by all of the populations of the PVG (except THY), indicating their relationships through nuclear genes. Thus, the genetic similarity of TAG and TEY possibly indicates an introgression between these adjoining populations. The second question concerns the divergent ASM, TAT and TKK populations of *P. venosus*. Although ASM was already suggested as a subspecies (*P. venosus zangezuri*; Stolyarov 1983), the small sample size prevents us from making any further decision. The other two populations exhibit some divergence phenetically but are still within *P. venosus* (Appendix S4&5; Kaya *et al.* 2013a). As they are isolated from the central population by the Aras River Basin, considering them as a candidate or a further bud species is logical.

The distribution pattern of the populations/species of the PVG is consistent with phenetic and genetic clustering, but is inconsistent with phylogenetic relationships (Fig. 2). The ranges of THY (*P. hakkari*), TVT (*P. tendurek*) and TGA (*P. rugulosus*) are disjoint from the central range of the PVG; the first two are in the southern margin and the third in the western margin of the PVG's range, and all are separated from the centre by lowlands. The TEY population of *P. rugulosus* is parapatric with TAG (*P. rugulosus*). Again, the Aras River Basin isolates the ranges of the divergent populations TAT, TKK (both from Aras Güneyi Mts) and ASM (from Syunik, Armenia) from the central range on the Ardahan Plateau. Of the remaining seven populations of *P. venosus*, GGC is distantly located from the core

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Table 6 The 95% highest posterior density (HPD) credibility intervals for the nodes numbered on the BEAST chronograms given in Figs 4 and 5

Genes	Calibration by rate				Calibration by age of Çoruh River (5.5 ± 0.2 Myr)					
	12S–16S (linked to COI) 95% HPD		COI 95% HPD		12S–16S 95% HPD			COI 95% HPD		
Nod	Relaxed		Strict	Relaxed	Strict		Relaxed	Strict		Relaxed
Psorodonotus	4.32	3.04–5.78	6.33	5.28–7.45	5.73	5.09–6.52	5.99	5.21–6.92	6.34	5.20–7.78	5.88	5.21–6.61	5.96	5.18–6.95
PCG+(*P. specularis* +PVG)	3.80	2.66–5.01	5.90	4.92–6.88	5.37	4.99–5.76	5.48	5.09–5.87	5.48	5.08–5.86	5.48	5.10–5.88	5.48	5.09–5.87
PCG	1.78	0.95–2.65	3.60	2.93–4.27	2.84	1.95–3.75	2.69	1.86–3.61	2.48	1.27–3.71	3.33	2.75–3.92	3.26	2.34–4.20
P. specularis +PVG	3.08	2.06–4.18	4.92	3.92–6.00	4.11	2.89–5.30	4.62	3.76–5.40	4.34	3.18–5.39	4.56	3.75–5.36	4.53	3.40–5.55
PVG	1.42	0.87–2.03	1.79	1.37–2.23	2.32	1.49–3.33	1.57	1.10–2.08	2.05	1.13–3.08	1.65	1.24–2.07	2.94	1.90–4.04
PVG-PhG1	0.89	0.50–1.31	0.96	0.72–1.21	1.32	0.79–1.95	1.04	0.66–1.43	1.29	0.65–2.05	0.88	0.64–1.13	1.80	1.00–2.67
PVG-PhG1a+*P. rugulosus*	0.69	0.32–1.08	0.59	0.39–0.80	0.88	0.42–1.42	0.82	0.45–1.21	1.00	–	0.54	0.35–0.74	1.21	0.55–1.96
PVG-PhG2	0.82	0.48–1.21	1.00	0.77–1.24	1.33	0.84–1.88	1.01	0.63–1.41	1.32	0.64–2.13	0.92	0.69–1.15	1.80	1.08–2.60
PVG-PhG2a+(*P. hakkari* + *P. tendurek*)	0.82	0.48–1.21	0.80	0.61–1.01	1.07	0.67–1.53	1.01	0.64–1.41	1.06	–	0.74	0.55–0.94	1.47	0.86–2.18
P. hakkari+*P. tendurek*	0.38	0.09–0.73	0.59	0.37–0.82	0.70	0.23–1.18	0.45	0.17–0.77	0.55	0.12–1.07	0.54	0.34–0.75	0.94	0.28–1.61
Bayes factors	−3005.056 ± 0.011		−6577.332 ± 0.166		−6562.834 ± 0.175		−3011.734 ± 0.103		−3003.789 ± 0.111		−6577.62 ± 0.172		−6562.059 ± 0.175	
Max_{ESS}–Min_{ESS}	16951–2065		21913–1136		10574–513		43888–2348		45001–1768		42481–1098		43504–400	

range and seems to be isolated by the intramontane Caucasian Basin. However, GGC is not genetically or phenetically divergent from typical *P. venosus* and possibly reached the Greater Caucasus recently. The remaining six populations of *P. venosus* are distributed on and around the Ardahan Plateau, which is located between Turkey, Armenia and Georgia. Thus, *P. venosus* occupies the central range of the PVG, and other species are isolated in the periphery, as expected for peripherally isolated populations/species (Mayr & Ashlock 1992; Frey 1993; Funk & Omland 2003; Coyne & Orr 2004).

Genetic diversity indices and results of demographic tests are congruent with the above-defined divergence and centre–periphery distribution patterns. *P. tendurek* and *P. hakkari* each have a single haplotype for each marker (Appendix S2). Genetic diversity indices of TAT and TKK are not zero but are low compared to other populations of *P. venosus*, and demographic tests suggested a contraction for each. The lowest diversity indices were recorded from the TGA population of *P. rugulosus*, although demographic tests suggested an expansion approximately 50 Kyr ago, possibly following the foundation event. The same pattern (low diversity indices) is valid for the peripheral Greater Caucasus (GGC) population of *P. venosus*, as its diversity indices are lower than those in the centre of the range. In contrast to these populations, diversity parameters are considerably high, and demographic tests suggested historically constant population sizes for all of the remaining populations of *P. venosus*, which are in the centre of the range. Thus, all of the results together are consistent with a history of speciation via peripheral isolation; genetic diversity is reduced and contains signs of founder effects/genetic drift in peripheral or bud species/populations, but not in central-ancestral species (Frey 1993; Coyne & Orr 2004).

From our perspective, '*an older ancestral species and younger periphery/bud species*' is the most demonstrative criterion for budding speciation. Only a gene tree with estimated divergence times may provide such evidence. The TMRCA for both the PVG and *P. venosus* is dated at approximately 2.05-2.94 Myr. The TMRCA for *P. tendurek* + *P. hakkari* is dated at approximately 0.55-0.94 Myr. Again, the TMRCA for PhG1A (including *P. rugulosus*) is dated at approximately 0.82–1.21 Myr. Thus, the molecular clock estimates for the PVG and its bud species are very consistent with the criterion. Additionally, molecular clock estimates indicate a strong correlation between the radiations within the PVG and the main climatic transitions in the Pleistocene (Fig. 6). The basal radiation within the PVG corresponds to the Gelacian/Calabrian transition when glacial cycles become prominent. Radiation within PhG1 and PhG2 correlates with the MPT when glacial cycles extended from 41 to 100 Kyr in Calabrian. The

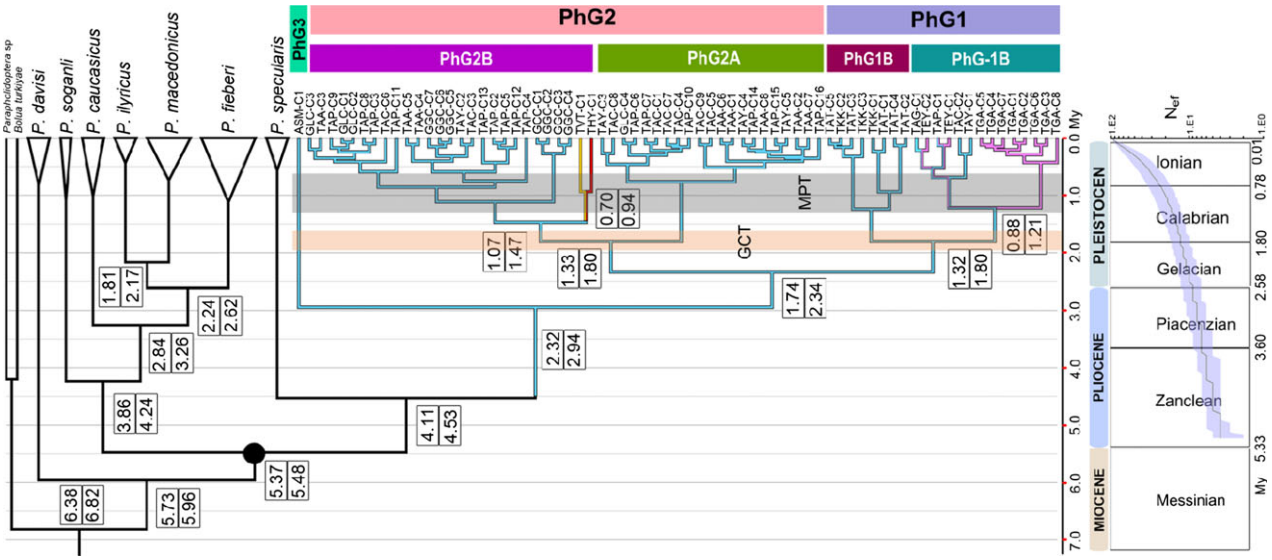

Fig. 5 BEAST chronogram of the COI data set for *Psorodonotus*. Divergence times were estimated according to Bayesian inference in BEAST (upper cell of the boxes along the nodes indicates means for the BEAST run calibrated using the substitution rate of 0.017 subs/s/Myr and the lower cells to runs calibrated to the age of Çoruh River, both under the relaxed clock option). The 95% highest posterior density (HPD) credibility intervals for the nodes numbered are given in Table 6.

Table 7 Estimated gene-specific divergence rates based on calibration by the age of Çoruh Valley setting the *P. specularis*+PVG node to 5.5 Myr (±0.2)

	Calibration by Çoruh Valley					
	Strict	Relaxed				
	Clock rate	Mean rate[a]	ucld.mean[b]	ucld.stdevc[c]	Coefficient variation[d]	Covariance[e]
12S-tDNAval-16S	0.0097 ± 8.03^{-6}	0.0119 ± 3.86^{-6}	0.0095 ± 3.14^{-5}	0.6068 ± 4.40^{-3}	0.6545 ± 5.71^{-3}	0.0484 ± 1.70^{-3}
COI	0.0187 ± 1.75^{-5}	0.0182 ± 9.44^{-5}	0.0116 ± 9.25^{-5}	0.8319 ± 5.03^{-3}	1.00 ± 9.46^{-3}	0.1042 ± 2.67^{-3}

[a]Number of substitutions per site divided by tree length.
[b]Mean of branch rates.
[c]The SD of the branch rates.
[d]Coefficient of variation.
[e]Covariance between parent and child branch rates.

divergence of bud species *P. tendurek*, *P. hakkari* and *P. rugulosus* was possibly triggered by intense glacial periods. Thus, combining the radiation history and ecological preferences of the group indicates the following scenario (Fig. 7). The Ardahan Plateau constituted the ancestral range. This ancestral population experienced range expansions and retractions due to climatic shifts in the Pleistocene. Following the range retractions, peripherally isolated remnants diverged into the present bud species on mountain summits, as assumed by the rear/leading edge concept (Çıplak 2004; Hampe & Petit 2005) and as observed in other Anatolian lineages (Sağlam *et al.* 2013; Korkmaz *et al.* 2014; Çıplak *et al.* 2015; Kaya *et al.* 2015,b). While peripheral isolates underwent these processes, the splitting and re-lumping of the ancestral populations prevented interruption of gene flow between central populations. This process increased and conserved the diversity in the centre while restricting the diversity of peripheral isolates (Frey 1993) (Fig. 7).

A potpourri of further indications in the data

In speciation processes, new species start from initial polyphyly and achieve monophyly through paraphyly (Avise 2000). This progress may be more rapid for mitochondrial

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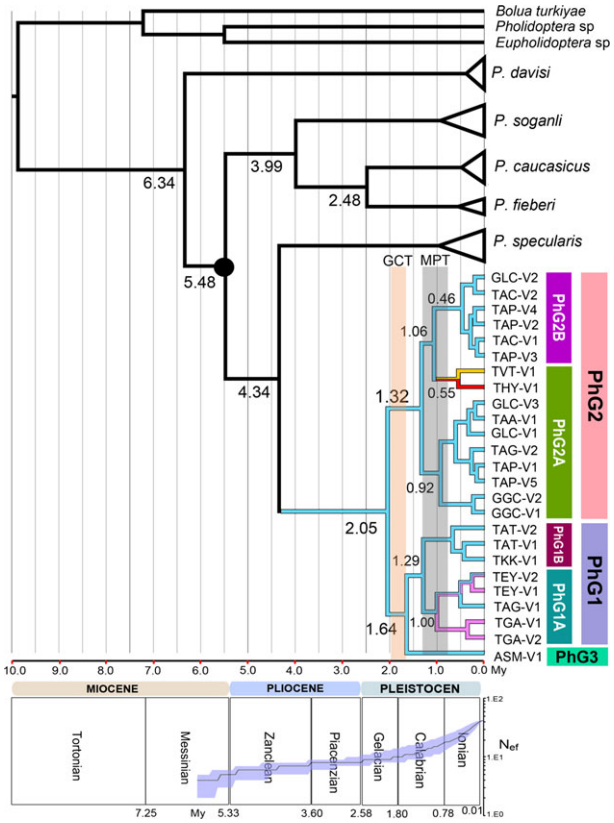

Fig. 6 BEAST chronogram of 12S-16S data set for *Psorodonotus*. Divergence times were inferred according to Bayesian inference in BEAST under relaxed clock option calibrated by the age of Çoruh River. The 95% highest posterior density (HPD) credibility intervals for the nodes numbered are given in Table 6.

haplotypes and can be rather distinctive in the case of peripheral isolates (Frey 1993; Funk & Omland 2003). The case of *P. rugulosus* (especially TEY) may violate this generalization because of sporadic genetic introgression between the ancestral and bud species. A delay in reaching monophyly is also probable for the ancestral species, as the gene flow at the central range is great enough to prevent the differentiation of central demes. Moreover, from a cladistic perspective (Hörandl & Stuessy 2010; Podani 2013), a gene tree including ancestral and bud species will remain paraphyletic until the ancestral species splits into further new species and has lost the characteristics of the budding species. Even so, full monophyly of an ancestral species may not be possible, as the ancestral-derived position of old and new alleles remains (Fig. 7).

The time estimation analyses allowed us to test previously introduced rates or to introduce new substitution rates for the markers studied. Substitution rate estimations for COI range from 0.0168 to 0.0177 subs/s/Myr for insects (for details, see Papadopoulou *et al.* 2010; Allegrucci *et al.* 2011). The only rate estimation for Tettigoniidae by Shapiro *et al.* (2006) was 0.015–0.02 subs/s/Myr, a range whose upper bound is higher than the common rates. BEAST analyses of the COI matrix calibrated by the age of Çoruh River produced slightly higher rates, such as 0.0187 (under strict clock option) and 0.0182 (under relaxed clock option) subs/s/Myr, but the BF for the second was higher. Although there are rate estimations for each of the 12S and 16S rRNA (0.011 and 0.007 subs/s/Myr, respectively; Allegrucci *et al.* 2011), estimates for total 12S rRNA-tDNAval-16S rRNA do not yet exist. BEAST analyses of 12S–16S suggested a rate of 0.0097 and 0.0119 subs/s/Myr under strict and relaxed clock model options, respectively. However, the BF for the second is higher.

The radiation history of PVG supports the glacial refugial position of North-east Anatolia and the Lesser Caucasus. Although most previous studies have emphasized the longitudinal range changes caused by climatic shifts (Hewitt 1996), the vertical range changes seem to be the main pattern for PVG. As for some other lineages (Tarkhnishvili *et al.* 2000; Seddon *et al.* 2002; Çıplak 2004; Sağlam *et al.* 2013), there are no representatives of the genus north of the Greater Caucasus. This mountain range may have prevented faunal communication between Anatolia and northern West Asia. However, climatic shifts caused range changes within the refugium, such as from the Caucasus to the Zagros Mountains latitudinally (which resulted in the foundation of *P. hakkari* and *P. tendurek*) or from the Caucasus to the Giresun Mountains longitudinally (which resulted in the foundation of *P. rugulosus*). Although the pioneers colonized suitable habitats in adjoining areas, some stocks always remained in the centre. The Posof Valley and adjoining highlands of Ardahan Plateau are the most probable local refugia for the long-term persistence of the PVG. The diversity indices and the haplotype composition of the TAP and TAY populations are consistent with this assumption (Appendix S2). Similarly, the low $gamma_{ST}$ values for the pairs forming between these two populations and others support the same pattern. Thus, altitudinal heterogeneity played a buffer role in long-term persistence during climate shifts (Kaya *et al.* 2012; Çıplak *et al.* 2015). Finally, the divergences of the PVG and other lineages of Anatolian origin during the Pleistocene point to a general pattern for the Anatolian refugium. The starting of glacial periods, the extension of the cold period from 41 to 100 kyr (MPT) and the initiation of four intense glacial periods are the main events that have triggered radiation here (Çıplak *et al.* 2015; Kaya *et al.* 2015b). These climatic events have increased biodiversity in the area in the way proposed by the rear/leading edge concept (Çıplak 2004; Hampe & Petit 2005; Çıplak *et al.* 2015) and have thus

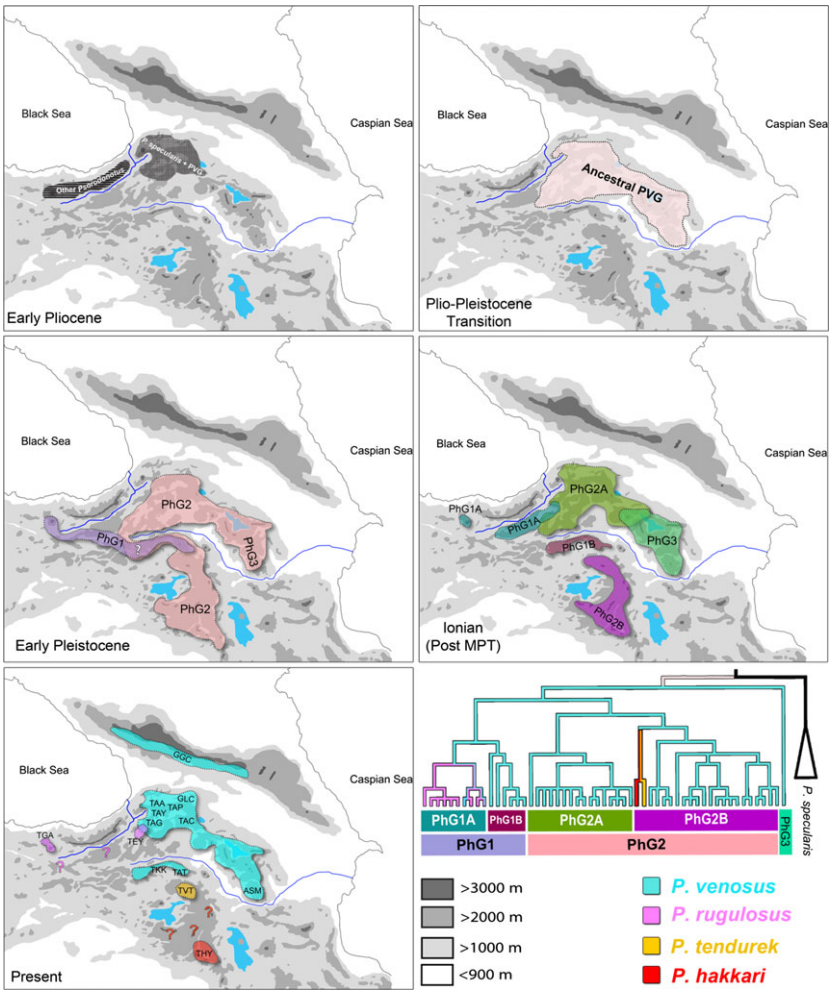

Fig. 7 The scenario proposed for speciation of PVG.

created a biodiversity hotspot in the Lesser Caucasus area (Fig. 8). The geological events prior to the Pleistocene, such as development of Çoruh Valley and volcanic events around the Ardahan Plateau (Sosson *et al.* 2010), were also important events that contributed to the biodiversity in the area. Ecological modelling of the lineages living in this area will provide further insights in this respect.

In the light of presented data and above statements, we concluded that *P. venosus* is the ancestral species and other three are bud species. In contrast to ancestral species, genetic diversity is reduced, and there are signs of departure from constant population size in bud species as expected under the budding speciation model. The bud species have isolated and restricted ranges, while the ancestral species does not. The relative ages of ancestral and bud species are the most reliable data to confirm the model, and chronograms of PVG well support this prediction.

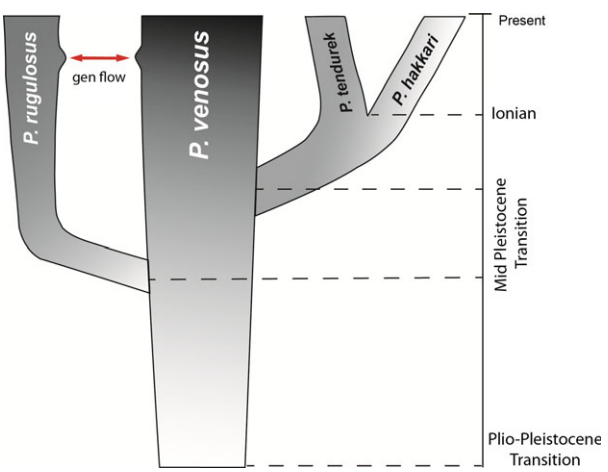

Fig. 8 Divergence model of PVG indicating ancestral and bud species.

The divergence events in the group co-occurred with major climatic transformations of the Pleistocene. There are secondary conclusions to be drawn from speciation pattern of PVG. First, although it is assumed that new species start from initial polyphyly and achieve monophyly through paraphyly, budding speciation violates this pattern. Second, the starting of glacial periods in Pliocene–Pleistocene, the extension of the cold period and the initiation of intense glacial periods are the main events that produced present species in Anatolio-Caucasian refugial system. Finally, development of Çoruh Valley seems an important geological event in south-east Black Sea Basin.

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Author contributions

B.Ç. conceived the ideas; B.Ç. and S.K. collected the data; S.K. analysed the data; and B.Ç led the writing.

References

Allegrucci, G., Trucchi, E. & Sbordoni, V. (2011). Tempo and mode of species diversification in *Dolichopoda* cave crickets (Orthoptera, Rhaphidophoridae). *Molecular Phylogenetics and Evolution*, *60*, 108–121.

Avise, J.C. (2000). *Phylogeography: the History and Formation of Species*. Massachusetts: Harvard University Press.

Blair, C., Heckman, K.L., Russell, A.L. & Yoder, A.D. (2014). Multilocus coalescent analyses reveal the demographic history and speciation patterns of mouse lemur sister species. *BMC Evolutionary Biology*, *14*, 57.

Butlin, R.K., Galindo, J. & Grahame, J.W. (2008). Sympatric, parapatric or allopatric: the most important way to classify speciation? *Philosophical Transaction of the Royal Society of London. Series B*, *363*, 2997–3007.

Butlin, R., Debelle, A., Kerth, C., Snook, R. R., Beukeboom, L. W., Castillo Cajas, R. F., Diao, W., Maan, M. E., Paolucci, S., Weissing, F. J., van de Zande, L., Hoikkala, A., Geuverink, E., Jennings, J., Kankare, M., Knott, K. E., Tyukmaeva, V. I., Zoumadakis, C., Ritchie, M. G., Barker, D., Immonen, E., Kirkpatrick, M., Noor, N., Garcia, C. M., Schmitt, T. & Schilthuizen, M. (2012). What do we need to know about speciation? *Trends in Ecology and Evolution*, *27*, 27–39.

Carstens, B.C., Pelletier, T.A., Reid, N.M. & Satler, J. (2013). How to fail at species delimitation? *Molecular Ecology*, *22*, 4369–4383.

Chan, K. & Moore, B. R. (2005). SymmeTREE: whole-tree analysis of differential diversification rates. *Bioinformatics*, *21*, 1709–1710.

Çıplak, B. (2004). Systematics, phylogeny and biogeography of *Anterastes* (Orthoptera, Tettigoniidae, Tettigoniinae): evolution within a refugium. *Zoologica Scripta*, *33*, 19–44.

Çiplak, B. (2008). The analogy between glacial cycles and global warming for the glacial relicts in a refugium: a biogeographic perspective for conservation of Anatolian Orthoptera. In S. Fattorini (Ed.) *Insect Ecology and Conservation* (pp. 135–163). Kerela: Research Signpost.

Çıplak, B., Kaya, S. & Gündüz, I. (2010). Phylogeography of *Anterastes serbicus* species group (Orthoptera, Tettigoniidae): phylogroups correlate with mountain belts, but not with the morphospecies. *Journal of Orthoptera Research*, *19*, 89–100.

Çıplak, B., Kaya, S., Boztepe, Z. & Gündüz, I. (2015). Mountainous genus *Anterastes* (Orthoptera, Tettigoniidae): autochthonous survival in refugial habitats across several glacial ages via vertical range shifts. *Zoologica Scripta*, *44*, 534–549.

Coyne, J.A. & Orr, H.A. (2004). *Speciation*. Sunderland, Massachusetts: Sinauer.

Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, *9*, 772.

Drummond, A. J., Rambaut, A., Shapiro, B. & Pybus, O.G. (2005). Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology Evolution*, *22*, 1185–1192.

Drummond, A.J., Suchard, M.A., Xie, D. & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7.*Molecular Biology And Evolution*, *29*, 1969–1973.

Eades, D.C., Otte, D., Cigliano, M.M. & Braun, H. (2015). *Orthoptera Species File*. Version 5.0/5.0. Available via http://Orthoptera.SpeciesFile.org

Excoffier, L. & Lischer, H.E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, *10*, 564–567.

Fenn, J.D., Song, H., Cameron, S.L. & Whiting, M.F. (2008). preliminary mitochondrial genome phylogeny of Orthoptera (Insecta) and approaches to maximizing phylogenetic signal found within mitochondrial genome data. *Molecular Phylogenetics and Evolution*, *49*, 59–68.

Fitzpatrick, B.M., Fordyce, J.A. & Gavrilets, S. (2009). Pattern, process and geographic modes of speciation. *Journal of Evolutionary Biology*, *22*, 2342–2347.

Frey, J.K. (1993). Modes of peripheral isolate formation and speciation. *Systematic Biology*, *42*, 373–381.

Fu, Y.X. (1997). Statistical tests of neutrality of mutations against population growth, hitch-hiking, and background selection. *Genetics*, *147*, 915–925.

Fu, Y. X. & Li, W. H. (1993). Statistical tests of neutrality of mutations. *Genetics*, *133*, 693–709.

Funk, D.J. & Omland, K.E. (2003). Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution and Systematics*, *34*, 397–423.

Gray, D.A., Huang, H. & Knowles, L.L. (2008). Molecular evidence of a peripatric origin for two sympatric species of field crickets (*Gryllus rubens* and *G. texensis*) revealed from coalescent

simulations and population genetic tests. *Molecular Ecology*, *17*, 3836–3855.

Hampe, A. & Petit, R. J. (2005). Conserving biodiversity under climate change: the rear edge matters. *Ecology Letters*, *8*, 461–467.

Harpending, H. (1994). Signature of ancient population growth in a low-resolution mitochondrial DNA mismatched distribution. *Human Biology*, *66*, 591–600.

Hennig, W. (1966). Translated by D.D. Davis & R. Zangerl. *Phylogenetic Systematics*. Urbana: University of Illinois Press.

Hewitt, G.M. (1996). Some genetic consequence of ice ages, and their role in diverging and speciation. *Biological Journal of the Linnean Society*, *58*, 247–276.

Hörandl, E. & Stuessy, T.F. (2010). Paraphyletic groups as natural units of biological classification. *Taxon*, *59*, 1641–1653.

Hudson, R.R., Boos, D.D. & Kaplan, N.L. (1992). A statistical test for detecting geographic subdivision. *Molecular Biology and Evolution*, *9*, 138–151.

Kaya, S., Gündüz, I. & Çıplak, B. (2012). Estimating effects of global warming from past range changes for cold demanding refugial taxa: a case study on South-west Anatolian species *Poecilimon birandi*. *Biologia*, *67*, 1152–1164.

Kaya, S., Korkmaz, E.M. & Çıplak, B. (2013a). *Psorodonotus venosus* group (Orthoptera, Tettigoniidae; Tettigoniinae): geometric morphometry revealed two new species in the group. *Zootaxa*, *3750*, 37–56.

Kaya, S., Boztepe, Z. & Çıplak, B. (2013b). Phylogeography of *Troglophilus* (Orthoptera: Troglophilinae) based on Anatolian members of the genus: radiation of an old lineage following the Messinian. *Biological Journal of the Linnean Society*, *108*, 335–348.

Kaya, S., Chobanov, D.P. & Çıplak, B. (2014). Review of *Psorodonotus specularis* group (Orthoptera, Tettigoniidae, Tettigoniinae): two new species from North-east Anatolia. *Zootaxa*, *3895*, 367–400.

Kaya, S., Chobanov, D.P., Skejo, L., Heller, K.-G. & Çıplak, B. (2015a). The Balkan *Psorodonotus* (Orthoptera: Tettigoniidae): testing existing taxa confirmed presence of three distinct species. *European Journal of Entomology*, *112*, 525–541.

Kaya, S., Boztepe, Z. & Çıplak, B. (2015b). Phylogeography of the *Poecilimon luschani* species group (Orthoptera, Tettigoniidae): a radiation strictly correlated to climatic transitions in the Pleistocene. *Zoological Journal of the Linnean Society*, *173*, 1–21.

Ketin, İ. (1976). San Andreas ve Kuzey Anadolu fayları arasında bir karşılaştırma. *Türkiye Jeoloji Bülteni*, *19*, 149–154.

Kim, S.I., Park, S.K., Lee, H., Oshida, T., Kimura, J., Kim, Y.J., Nguyen, S.T., Sashika, M. & Min, M.S. (2013). Phylogeography of Korean raccoon dogs: implications of peripheral isolation of a forest mammal in East Asia. *Journal of Zoology*, *290*, 225–235.

Knowles, L.L. & Carstens, B.C. (2007). Delimiting species without monophyletic gene trees. *Systematic Biology*, *56*, 887–895.

Korkmaz, E. M., Lunt, D. H., Çıplak, B., Değerli, N. & Başıbuyuk, H. H. (2014). The contribution of Anatolia to European phylogeography: the centre of origin of the meadow grasshopper, *Chorthippus parallelus*. *Journal of Biogeography*, *41*, 1793–1805.

Kruckenhauser, L., Duda, M., Bartel, D., Sattmann, H., Harl, J., Kirchner, S. & Haring, E. (2013). Paraphyly and budding speciation in the hairy snail (Pulmonata, Hygromiidae). *Zoologica Scripta*, *43*, 273–288.

Losos, J.B. & Glor, R.E. (2003). Phylogenetic comparative methods and the geography of speciation. *Trends in Ecology & Evolution*, *18*, 220–227.

Losos, J.B. & Ricklefs, R.E. (2009). Adaptation and diversification on islands. *Nature*, *457*, 830–836.

Mayr, E. & Ashlock, P.D. (1992). *Principles of Systematic Zoology*, 2nd edn. Oxford: Oxford University Press.

Minin, V.N., Bloomquist, E.W. & Suchard, M.A. (2008). Smooth skyride through a rough skyline: Bayesian coalescent-based inference of population dynamics. *Molecular Biology and Evolution*, *25*, 1459–1471.

Papadopoulou, A., Anastasiou, I. & Vogler, A.F. (2010). Revisiting the insect mitochondrial molecular clock: the Mid-Aegean Trench calibration. *Molecular Biology and Evolution*, *27*, 1659–1672.

Patino, J., Carine, M., Fernandez-Palacios, J.M., Otto, R., Schaefer, H. & Vanderpoorten, A. (2014). The anagenetic world of spore-producing land plants. *New Phytologist*, *201*, 305–311.

Podani, J. (2013). Tree thinking, time and topology: comments on the interpretation of tree diagrams in evolutionary/phylogenetic systematics. *Cladistics*, *29*, 315–327.

Ramos-Onsins, S. E. & Rozas', J. (2002). Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, *19*, 2092–2100.

Rogers, A.R. & Harpending, H. (1992). Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, *9*, 552–569.

Rozas, J. (2009). DNA sequence polymorphism analysis using DnaSP. In D. Posada (Ed.) *Bioinformatics for DNA Sequence Analysis; Methods in Molecular Biology Series*, Vol. 537 (pp. 337–350). New Jersey: Humana Press.

Sağlam, İ.K., Küçükyıldırım, S. & Çağlar, S.S. (2013). Diversification of montane species via elevation shifts: the case of the Kaçkar cricket *Phonochorion* (Orthoptera). *Journal of Zoological Systematics and Evolutionary Research*, *52*, 177–189.

Sarıkaya, M.A., Çiner, A. & Zreda, M. (2011). Quaternary glaciations of Turkey. In J. Ehlers, P.L. Gibbard & P.D. Hughes (Eds) *Developments in Quaternary Science*, Vol. 15, (pp. 393–403). Amsterdam: Elsevier.

Schneider, S. & Excoffier, L. (1999). Estimation of demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. *Genetics*, *152*, 1079–1089.

Seddon, J.M., Santucci, F., Reeve, N. & Hewitt, G.M. (2002). Caucasus Mountains divide postulated postglacial colonization routes in the white-breasted hedgehog, *Erinaceus concolor*. *Journal of Evolutionary Biology*, *15*, 463–467.

Shapiro, L.H., Strazanac, J.S. & Roderick, G.K. (2006). Molecular phylogeny of *Banza* (Orthoptera: Tettigoniidae), the endemic katydids of the Hawaiian Archipelago. *Molecular Phylogenetics and Evolution*, *41*, 53–63.

Sosson, M., Kaymakçı, N., Stephenson, R., Bergerat, F. & Starostenko, V. (Eds) (2010). *Sedimentary Basin Tectonics from the Black Sea And Caucasus to the Arabian Platform*, Vol 340. Special Publications, Geological Society: London.

Stolyarov, M.V. (1983). New data on the Orthoptera of the Caucasus and Turkey. *Entomologicheskoe Obozrenie*, *62*, 501–511.

Stuessy, T.F., Jakubowsky, G., Gomez, R.S., Pfosser, M., Schluter, P.M., Fer, T., Sun, B.-Y. & Kato, H. (2006). Anagenetic evolution in island plants. *Journal of Biogeography*, *33*, 1259–1265.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, *123*, 585–595.

Takayama, K., Sun, B.-Y. & Stuessy, T.F. (2013). Anagenetic speciation in Ullung Island, Korea: genetic diversity and structure in the island endemic species, *Acer takesimense* (Sapindaceae). *Journal of Plant Research*, *126*, 323–333.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*, 2725–2729.

Tarkhnishvili, D., Thorpe, R.S. & Arntzen, J.W. (2000). Pre-Pleistocene refugia and differentiation between populations of the Caucasian salamander (*Mertensiella caucasica*). *Molecular Phylogenetics and Evolution*, *14*, 414–422.

Tedesco, P.A., Leprieur, F., Hugueny, B., Brosse, S., Dürr, H.H., Beauchard, O., Busson, F. & Oberdorff, T. (2012). Patterns and processes of global riverine fish endemism. *Global Ecology and Biogeography*, *21*, 977–987.

Toussaint, E.F.A., Sagata, K., Surbakti, S., Hendrich, L. & Balke, M. (2013). Australasian sky islands act as a diversity pump facilitating peripheral speciation and complex reversal from narrow endemic to widespread ecological supertramp. *Ecology and Evolution*, *3*, 1031–1049.

Supporting Information

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

Appendix S1. Supplements to Materials and Methods.

Appendix S2. Shared ITS1-5.8S ITS2 (2A), COI (2B) and rRNA-tRNAval-16S rRNA (2C) haplotypes between populations and species.

Appendix S3. Phylogenetic analyses and range shift tests for COI and 12S–16S data sets of *Psorodonotus* using SymmeTREE.

Appendix S4. Geometric morphometric analyses applied to male cerci (A) and ovipositor (B) of the PVG.

Appendix S5. Male calling songs of the PVG species.