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Phylogeography of the *Poecilimon luschani* species group (Orthoptera, Tettigoniidae): a radiation strictly correlated with climatic transitions in the Pleistocene

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The Quaternary biogeography of Anatolia has received considerable interest recently. Here, the genealogical history of the Anatolio–Balkan lineage of the *Poecilimon luschani* species group was evaluated. Using concatenated data from 16S rDNA and cytochrome c oxidase subunit I (COI) sequences, the timings of inter- and intraspecies radiations were estimated. The demographic history of the populations was estimated using a data set established from COI sequences. Genetic diversity was very high in almost all of the populations studied. Fixation indices suggested extreme divergence of *P. luschani*. A molecular chronogram estimated a radiation history for the species/subspecies over a period ranging from 1.323 to 0.440 Myr. Demographic analyses applied to 11 populations suggested departure in population size for most of the local populations. The following conclusions were reached: (1) *P. luschani* originated from an Anatolio-Aegean ancestral stock and extended its range to the Balkans through Dardanelles during the Early Pleistocene; (2) the Mid-Pleistocene Transition, the lengthening of glacial period from 41 to 100 Kyr and the initiation of intense glaciation periods are the three main events corresponding to the main nodes of the chronogram; (3) altitudinal heterogeneity played a buffer role during the glacial cycles, allowing populations to cope with severe environmental changes; (4) the effects of Pleistocene climate cycles on populations differ according to altitudinal and latitudinal location in Anatolia, and (5) habitat preferences, such as altitudinal range, may easily shift because of changes in environmental conditions.

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INTRODUCTION

Anatolian biogeography has received considerable interest recently because of several features of the region. Some of those most frequently stated are that: (1) it has rich and endemic biodiversity (Myers *et al*., 2000; Çıplak, 2003; Médail & Diadema, 2009; Şekercioğlu *et al*., 2011); (2) it was/is a dispersal corridor between Asia, Europe, and Africa (Kosswig, 1955); (3) it was a significant refugium during the glacial ages of the Quaternary (Hewitt, 1996; Çıplak, 2004, 2008; Bilgin, 2011) and its connections with the Balkans and Caucasus were the main corridors allowing faunal/floral exchange during these climatic fluctuations (Korkmaz *et al*., 2014); and (4) its topography and climate are highly heterogeneous, resulting in significant habitat diversity (Çıplak, 2008; Bilgin, 2011; Şekercioğlu *et al*., 2011). However, these are very general statements and the full picture requires examination of each in more detail.

Anatolian biodiversity evolved under successive historical geological/geographical events, but the recent ones may have had greater effects than those occurring earlier (Çıplak, 2008). The successive climatic fluctuations of the Quaternary are possibly the main determinants of the distributions of present populations/taxa. Generalizations about effects of the glacial ages mainly concern postglacial northern expansion of the some Anatolian form (Hewitt, 1996; Rokas *et al*., 2003; Çıplak, 2004, 2008; Bilgin, 2011). In light of recent

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studies it has become apparent that intrarefugial range changes and speciation during the climatic cycles of the Quaternary exhibit different patterns depending upon the geographical sections and ecological preferences of the lineages under study. Some lineages experienced Pleistocene climate cycles in their present range without long dispersals out of their present distribution area, such as Lycian salamanders in the humid lowlands of the south-east corner of Anatolia (Veith *et al*., 2008), the Caucasian salamander and three species of *Phonochorion* in their local refugia in the north-east corner of Anatolia (Tarkhnishvili, Thorpe & Arntzen, 2000; Sağlam, Küçükyıldırım & Çağlar, 2013), the Anatolian members of the cave crickets *Troglophilus* in Mediterranean Anatolia (Kaya, Boztepe & Çıplak, 2013) and the bush cricket *Anterastes* in the western half of Anatolia (Çıplak, Kaya & Gündüz, 2010). By contrast, the range shift patterns defined for the ground squirrel *Spermaphilus* (Gündüz *et al*., 2007), tree frogs *Hyla* (Gvoždik *et al*., 2010), terrapins *Mauremys* (Fritz *et al*., 2008), gall wasp *Andricus caputmedusae* (Mutun, 2010), and the plant *Arabis* (Ansell *et al*., 2011) seem to be different in the western and eastern halves of Anatolia possibly because of the Anatolian Diagonal, which provides a south–north range corridor in the east, and the absence of such a prominent geographical entity in the west (Davis, 1971; Çıplak, Demirsoy & Bozcuk, 1993; Çıplak, 2008).

In addition to above taxa, some other lineages have changed their range from Anatolia to the southern surrounding areas such as the Levant, Arabian plate, and Iran (Çıplak & Heller, 2005; Challis *et al*., 2007). However, taxa of Anatolian origin, such as *Chorthippus paralellus*, have also managed to extend to large parts of Europe during the last four glacial periods, possibly more than once (Korkmaz *et al*., 2014). Apart from the estimated range change patterns, the time spans of divergence patterns differ significantly amongst the lineages. Some of these lineages have radiated to several taxa during the Middle and Late Pleistocene (Veith *et al*., 2008), whereas some others diverged only to some genetic subunit (Tarkhnishvili *et al*., 2000; Gündüz *et al*., 2007; Akın *et al*., 2010; Çıplak *et al*., 2010; Ansell *et al*., 2011). Diversity in estimating Quaternary range shift patterns indicates that Anatolia exhibits complex biogeography and robust patterns for this part of the Mediterranean, thus for the west Palaearctic, requires studying different lineages that differ in their geographical ranges and ecological preferences.

Poecilimon Fischer is a genus that ranges over the circum Black Sea and the Eastern Mediterranean Basins. It contains about 150 species that form several species groups (Çıplak, 2004; Eades *et al*., 2014). The *Poecilimon luschani* species group was recently revised by Boztepe, Kaya & Çıplak (2013) and includes eight taxa ranged along a narrow line in west Anatolia and the Rhodopian mountains in the Balkans. The phylogeny of these taxa is suggested to be *Poecilimon ledereri* + (*Poecilimon orbelicus* + (*Poecilimon tuncayi* + (*Poecilimon egrigozi* + *Poecilimon helleri*) + (*P. luschani luschani* + *Poecilimon luschani birandi* + *Poecilimon luschani chobanovi*))) (Boztepe *et al*., 2013). Previous studies on the group (Çıplak *et al*., 2008; Kaya, Gündüz & Çıplak, 2012; Boztepe *et al*., 2013) have indicated that this lineage exhibits some interesting aspects to be studied phylogeographically and to test the assumptions about possible effects of climatic fluctuations on Anatolian biodiversity specifically, and on intrarefugial populations/taxa in general. First, the present range of the linage is divided by the Turkish Strait System (Dardanelles + Marmara Sea + Bosphorus). Second, the species in the group occur over a wide range of altitudinal heterogeneity. Those in the northern part of the group's range are confined to some summits: *P. orbelicus* (Rhodopian Mountains in the Balkans), *P. egrigozi* (Egrigöz Mt. in north-west Anatolia), and *P. helleri* (Kazdag Mountain in north-west Anatolia). Two species in Aegean Anatolia, *P. ledereri* and *P. tuncayi*, occur in lowlands or moderate highlands, whereas *P. luschani* occurs along a wide altitudinal range from sea level up to 2250 m in the south-west corner of Anatolia. Third, *P. luschani* exhibits complex taxonomical and geographical-genetic structuring (Kaya *et al*., 2012; Boztepe *et al*., 2013). All of these features of the lineage indicate that it is a candidate model group to address whether or not climatic fluctuations have affected the southern and northern populations in different ways.

The present study aimed to address the following issues. Using molecular clock estimation, we first aimed to determine when the species group radiated. Having a time estimation allowed us to correlate speciation events with past geographical/climatic events. As the populations/taxa in the lineage exhibit different ecological preferences, assuming presence in the highlands as one character state and in the lowlands as the reverse state, a chronogram allowed us to test whether there is a correlation between the climatic shifts in the Quaternary and ecological shifts in the preferences of populations/taxa. The answer to this question and the historical demography provided an opportunity to evaluate whether the shift in ecological preferences allowed populations to cope with the changing climate and if altitudinal heterogeneity played a buffer role in this. The present range of the species group is divided by the Turkish Strait System. Determining the time of this separation made it possible to discuss the possible faunal exchanges between Anatolia and the Balkans during glacial periods. However, the *P. luschani* complex occurring in the south-west corner of Anatolia requires closer consideration as do some of the other lineages also occurring here (Veith

et al., 2008; Akın *et al*., 2010). Demographic analyses at the population level allowed us to define the local effects of the climate changes.

MATERIAL AND METHODS

Populations, data preparation and descriptive genetic

The *P. luschani* species group includes six species: *P. ledereri*, *P. orbelicus*, *P. tuncayi*, *P. egrigozi*, *P. helleri*, and *P. luschani*. The last, referred to as the *P. luschani* complex here after, includes three subspecies: *P. luschani luschani*, *P. luschani birandi*, and *P. luschani chobanovi* (Boztepe *et al*., 2013). In total, 16 populations representing all of these eight taxa were examined (Table 1, Fig. 1). The 16S rDNA and cytochrome c oxidase subunit I (COI) sequences, either unique haplotypes that were previously uploaded to a public medium (see Supporting Information Table 3; published in Kaya *et al*., 2012; Boztepe *et al*., 2013), or were newly obtained were used. The sequences of each marker were aligned in SEQUENCHER v. 4.1 (Gene Codes Corporation) and checked manually by eye separately. Unique haplotypes and their frequencies were determined using DnaSP v.5 (Librado & Rozas, 2009). The haplotype matrix was prepared using the Clustal-W option in MEGA v. 5 (Tamura *et al*., 2011). The COI sequences were checked for nuclear copies of mtDNA (numts) by examining chromatograms for double signals, by translating all fragments into amino acid sequences, and by looking for stop codons or indels. After an incongruence length difference (ILD) test (Farris *et al*., 1994) using PAUP v. 4.0b10 (Swofford, 2000), the 16S rDNA and COI sequences were concatenated into a final data matrix to be used in the time estimation analyses.

For demographic analyses and descriptive genetics of the populations, the downloaded sequences of unique haplotypes were used together with the other sequences in our database (for molecular procedures see Boztepe *et al*., 2013). The diversity parameters, such as the number of unique haplotypes (k, K), haplotype diversity ($h \pm$ SD), number of segregating sites (s), nucleotide diversity ($\pi \pm$ SD), and mean number of pairwise differences between k haplotypes ($d \pm$ SD), were estimated for the COI data using ARLEQUIN v. 3.5.1.2 (Excoffier & Lischer, 2010). For 16S rDNA sequences, only the number of unique haplotype is given.

Time estimation analyses

We applied two different approaches, the branch length differences method (Nee, 2001) and the topological method (Kirkpatrik & Slatkin, 1993), to our data set in order to determine whether or not it shows clock-like evolution. A maximum likelihood clock test (MLC), which depends on branch length differences on the tree (Tamura & Nei, 1993), was performed using MEGA. Diversification rate and rate shifts based on the topological method were tested using SymmeTREE v. 1.1 (Chan & Moore, 2005). SymmeTREE was applied to maximum parsimony (MP) bootstrap, maximum likelihood (ML), and Bayesian inference phylogeny (BIP) trees (given in Boztepe *et al*., 2013) separately. SymmeTREE was employed to conduct whole-tree tests for diversification rate variation using M-statistics [the cumulative equal-rates Markov probability by the product (M_Π) and sum (M_Σ) of individual nodal probabilities] (Chan & Moore, 2002; Moore, Chan & Donoghue, 2004), the Slowinski–Guyer statistic Δ_1/Δ_2 likelihood rate shift statistics to locate shifts (Slowinski & Guyer, 1989a, b), and Shao & Sokal Index B_1 to assess tree imbalance (Colless, 1982; Shao & Sokal, 1990; Heard, 1992). The diversification rate shifts analyses were conducted with 1 000 000 random resolutions and 10 000 for individual nodes, using the taxon-size sensitive equal rate Markov algorithm and 10^5 simulations settings for a strict consensus tree of 1007 equally parsimonious trees, ML tree, and BIP tree (given in Boztepe *et al*., 2013) separately.

The divergence time points between populations and clades were also estimated under a Bayesian statistical framework with BEAST v. 1.7.0 (Drummond & Rambout, 2007), using a data matrix concatenated from unique sequences of COI and 16S rDNA. Inferring from the results of the MLC and SymmeTREE, BEAST was run with different clock models. The Bayes factor (BF) (Kass & Raftery, 1995) was used to evaluate robustness of the models and the model with the highest BF was selected. For tree searching, the Yule tree prior, which assumes a constant speciation rate per lineage was employed. The time to the most recent common ancestor (TMRCA) between each phylogroup was estimated under the model parameters highlighted in MODELTEST v. 3.6 (Posada & Crandall, 1998). BEAST was run using two independent Markov chain Monte Carlo (MCMC) chains for 9×10^7 generations, sampling every 1000 generations. TRACER v. 1.5 (Rambout & Drummond, 2003) was used to monitor the convergence to stationary and the effective sample size of the model parameters. The maximum clade-credibility trees were produced with TREEANNOTATOR (Drummond & Rambout, 2007), discarding the initial 10% of samples as burn-in. The results, including confidence intervals, were visualized using FIGTREE 1.3.1 (Rambout, 2008).

As there are no available fossils of the genus or prominent vicariant events of the taxa to be used for calibration, the divergence time was estimated using the substitution rate for the mitochondrial genome. Substitution rate estimations for COI range from 0.0168 to 0.023 per site/million years (Brower, 1994; Papadopoulou, Anastasiou & Vogler, 2010; Allegrucci & Trucchi & Sbordoni *et al*., 2011). Even so, there are extreme rate estimations such as 0.03–0.04 per site/

Table 1. Sampling details (see Fig. 1) of specimens used in the study

Species	Population	Coordinates	Altitude (m)	Vegetation	Collection dates
Poecilimon ledereri	Turkey: İzmir, Bozdağ Mountain	38°17′39.2″N 028°01′06.6″E	768	High grasses	06/06/2010
Poecilimon orbelicus	Bulgaria: Pirin Mountain	41°48′16.0″N 028°08′59.4″E	2070	Alpine	24/08/2011
Poecilimon tuncayi	Turkey: Aydın, Çine	37°25′04.4″N 028°08′06.0″E	77	High grasses	05/06/2010, 15/05/2011
Poecilimon egrigozi	Turkey: Kütahya, Eğrigöz Mountain	39°24′10.1″N 029°08′46.3″E	1698	Alpine	04/07/2011
Poecilimon helleri	Turkey: Balıkesir, Kazdağı Mt.	39°42′19.6″N 026°52′44.3″E	1688	Alpine	05/07/2011
Poecilimon luschani chobanovi	Turkey: Antalya, Akdağ Mountain*	36°34′38.3″N 029°34′59.2″E	2247	Alpine	15/07/2005
	Turkey: Antalya, Erentepe Mountain*	36°44′38.3″N 029°38′45.0″E	1982	Alpine	15/07/2005
	Turkey: Antalya, Eşen Çayı 1	36°42′01.6″N 029°24′59.0″E	672	Maquis opening	15/05/2005, 14/05/2011
	Turkey: Antalya, Eşen Çayı 2	36°33′29.4″N 029°25′17.4″E	470	Maquis opening	08/05/2005, 14/05/2011
	Turkey: Antalya, Eşen Çayı 3	36°25′33.5″N 029°16′24.1″E	85	Maquis opening	20/05/2006, 14/05/2011
Poecilimon luschani luschani	Turkey: Antalya, Demre	36°16′32.1″N 029°56′36.8″E	120	Maquis opening	19/05/2006, 14/05/2011
	Turkey: Antalya, Kalkan	36°15′43.7″N 029°27′55.1″E	857	Maquis opening	15/05/2005, 13/05/2011
	Turkey: Antalya, Patara	36°17′22.5″N 029°20′03.5″E	110	Maquis opening	12/04/2007, 14/05/2011
Poecilimon luschani birandi	Turkey: Antalya, Termessos	36°35′34.0″N 030°16′49.1″E	878	Maquis – timberline	11/06/2005
	Turkey: Antalya, Bakırlıdağ Mountain	36°49′09.6″N 030°21′19.1″E	1819	Alpine	03/07/2004
	Turkey: Antalya, Olimpos	36°27′05.2″N 030°25′41.5″E	398	Maquis –timberline	19/05/2006, 13/05/2011
	Turkey: Antalya, Kemer	36°22′40.0″N 030°15′06.2″E	1110	Subalpine	02/06/2006
	Turkey: Antalya, Tahtalıdağ Mountain*	36°19′02.4″N 030°15′03.1″E	1850	Subalpine	10/07/2006
Outgroups					
Poecilimon obtusicercus	Turkey: Denizli, Tavas	37°22′27.0″N 029°08′14.0″E	1175		07/06/2010
P. obtusicercus	Turkey: Antalya, Korkuteli	36°56′06.8″N 030°05′49.4″E	1309		08/06/2010
Poecilimon cervus	Turkey: Bolu, Yedigöller	40°58′48.0″N 031°33′16.0″E	499		08/07/2011
Poecilimon izmirensis	Turkey: Kütahya, Eğrigoz Mountain	39°24′10.1″N 029°08′46.3″E	1698		04/07/2011

*sequences of cytochrome c oxidase subunit I were not available.

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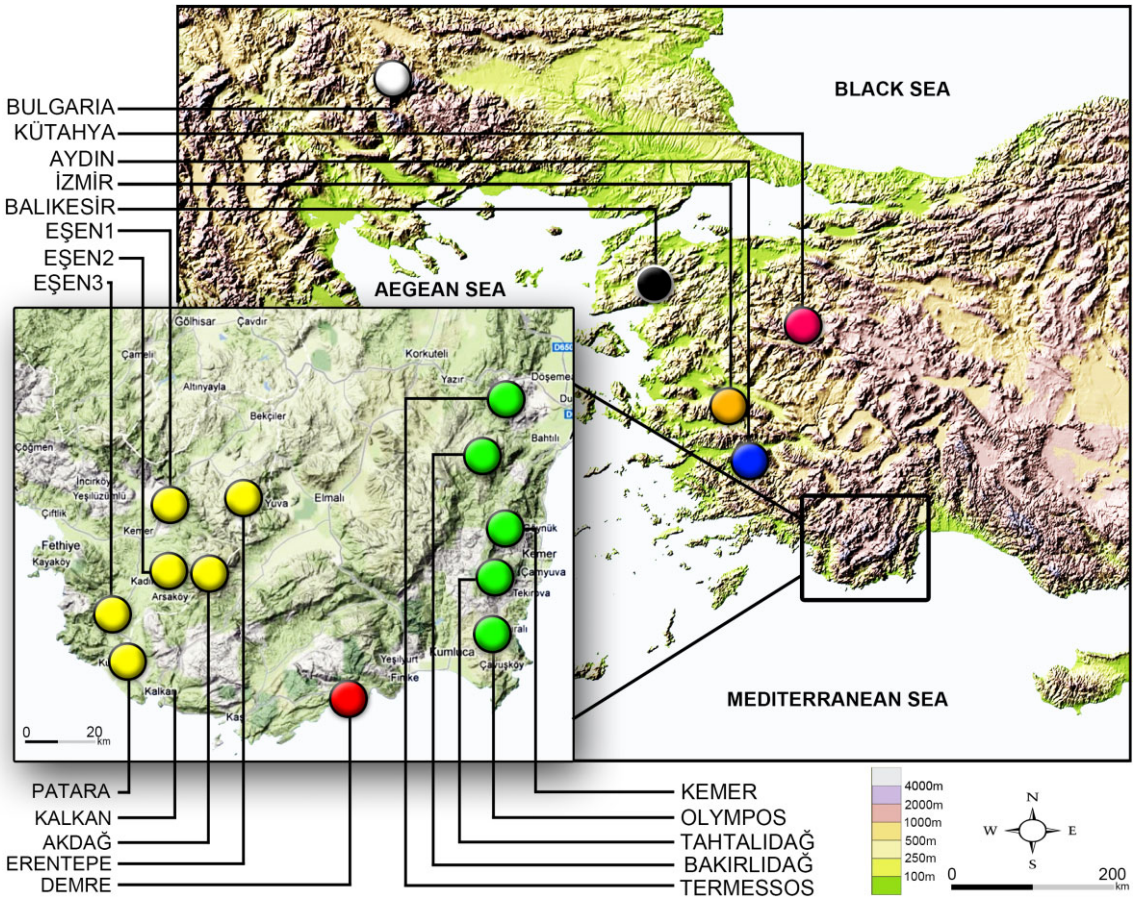

Figure 1. Populations sampled for specimens used in the study (for details of the localities see Table 1).

million years for Tettigoniidae (Shapiro, Strazanac & Roderick, 2006). The substitution rate per site/million years varies from 0.00172 to 0.0258 in eukaryotes (Kasuga, White & Taylor, 2002; Percy, Page & Cronk, 2004; Bargues *et al*., 2006). The substitution rate estimations for 16S or 12S–16S in insects vary between 0.0054 and 0.011 (Brower, 1994; Papadopoulou *et al*., 2010; Allegrucci, Trucchi & Sbordoni, 2011). In conclusion, it was more appropriate to calibrate the molecular clock analysis of the concatenated data set according to the general substitution rate for the insect mitochondrial genome suggested by Brower (1994; 0.023 substitutions/site/year), as widely used in similar studies (Percy *et al*., 2004; Kiyoshi & Sota, 2006; Papadopoulou *et al*., 2010; Allegrucci *et al*., 2011). The BEAST analysis was constrained with the tree topology given in Boztepe *et al*. (2013: fig. 85) for the nodes to species or earlier.

Demographic analyses

As each method has limits and pitfalls, different analyses were applied to estimate the demographic histories of populations using the data matrix of COI sequences. Changes through time in effective population size were evaluated with the Gaussian Markov random field (GMRF) skyride plots using BEAST v. 1.7.2. The GMRF skyride method, which uses the MCMC process, provides a general profile of population demographic fluctuation (Minin, Bloomquist & Suchard, 2008). The MCMC analysis of each run was performed with 30–40 × 10^6 generations (sampled every 1000 iterations), of which the first 10% was discarded as burn-in. TRACER v. 1.5 was used to estimate the convergence of chains, effective sample size (ESS) estimates and credible intervals for each parameter, and demographic reconstructions. In addition, the pairwise differences between sequences were examined using the mismatch distribution method (Rogers & Harpending, 1992; Harpending, 1994). Mismatch graphs are expected to be unimodal for populations that have undergone a recent expansion, but multimodal for populations at equilibrium or that have experienced genetic admixture in the past (Rogers & Harpending, 1992; Schneider & Excoffier, 1999). Mismatch analysis was conducted per population using

ARLEQUIN v. 3.5.1.2 (Excoffier & Lischer, 2010) under a model of population expansion. The overall validity of the estimated demographic model was evaluated by the tests of raggedness index (Hri) and the sum of squared differences (SSD). The significance of Hri and SSD was assessed by parametric bootstraps (10 000 replicates), and a significant value was taken as evidence for departure from the estimated demographic model of sudden population expansion.

In addition to skyride and mismatch analyses, Tajima's *D* (Tajima, 1989), Fu's *Fs* (Fu, 1997), Fu and Li's *D** (Fu & Li, 1993), and Ramos–Onsins and Rozas's R_2 (Ramos-Onsins & Rozas, 2002) were applied to test the selective neutrality of genetic markers. These estimators are also sensitive to demographic processes such as recent population expansion or bottlenecks. Of these estimators Tajima's *D*, Fu's *Fs* and R_2 are sensitive to an excess of recent mutations whereas Fu and Li's *D** can detect an excess of old mutations in a population that has experienced a historical reduction in effective population size. For each population, Tajima's *D* and Fu's *Fs* were estimated in ARLEQUIN v. 3.5.1.2 with 10 000 simulations, and Fu and Li's *D** and Ramos-Onsins and Rozas's R_2 tests were performed in DnaSP v. 5 with 1000 coalescent replicates. Finally, the exponential growth rate (*g*) for the populations was tested using a coalescent-based approach. The *g* parameter was estimated by the following strategy using LAMARCK v. 2.1.2 (Kuhner, 2006): initial chains; ten short chains of 1000 steps and final chains; two long chains of 20 000–40 000 steps, sampling every 20 steps, and a burn-in of 1000 trees.

RESULTS

Descriptive genetics

After alignment and trimming, the final length of 16S rDNA was 539 bp for the 249 samples from populations of the ingroup other than *P. egrigozi* (we were unsuccessful in amplifying 16S rDNA from specimens of the Kütahya population of this species with the primer pair used for the other specimens and so a shorter segment of this marker was amplified from two specimens of this population using another primer pair; see Boztepe *et al*., 2013). Of the 539 sites, 441 were invariable and 98 were variable, and of the variable sites 20 were indels. From the sequence analyses, 63 haplotypes belonging to seven ingroup taxa were identified amongst the 249 samples. The number of unique haplotypes within the ingroup reduced to 39 when the 20 indel sites were deleted. Almost all of the unique haplotypes is unique to the population in which it was observed (Table S1). When the above samples were aligned and trimmed together with two sequences of *P. egrigozi*, the final length of 251 sequences was 310 bp. Of the 310 sites, 248 were invariable and 62 were variable, and of the variable sites ten were indels. Fifty-one unique haplotypes were defined from these sequences and the number of unique haplotypes reduced to 30 when the ten indel positions were deleted (Table S1). Of these, 24 haplotypes were used to establish the concatenated data set used in the BEAST analysis (Table S3). Diversity indices based on this marker for the *P. luschani* complex were published in Kaya *et al*. (2012).

After alignment and trimming, the final length of COI was 1102 bp for the 234 sequences from eight ingroup taxa. All sequences of COI were checked for possible numt sequences using different approaches, but no sign of these was encountered. Of the 1102 sites, 828 were invariable and 274 were variable, of which 262 were parsimony informative. From the sequence analyses, 101 unique haplotypes were identified. No haplotype was shared by two or more species. There are a few haplotypes shared by different populations of the *P. luschani* complex: (1) haplotypes Kalkan4 and Kalkan5 between the Kalkan and Patara populations and (ii) haplotype Bakırlıdağ1 between the Bakırlıdağ and Termessos populations (Table S2). Sixty-nine unique haplotypes were used to establish the concatenated data matrix used in the BEAST analysis (Table S3).

Genetic diversity indices for the COI marker were calculated for 16 populations (Table 2). Haplotype diversity ranged between 0.1333 and 0.9560. The lowest values were displayed by the Balkan – *P. orbelicus* (0.1333), Bakırlıdağ – *P. luschani birandi* (0.1333), and Eşen1 – *P. luschani chobanovi* (0.2821) populations, whereas the highest values were found in the Eşen2 – *P. luschani chobanovi* (0.9560), İzmir – *P. ledereri* (0.9457), and Eşen3 – *P. luschani chobanovi* (0.9333) populations. Nucleotide diversity varied between 0.000120 and 0.017382. The lowest values were found in the Balkan – *P. orbelicus* (0.000120), Eşen1 – *P. luschani chobanovi* (0.000254), and Termessos – *P. luschani birandi* (0.000451) populations, whereas the highest values were present in the Demre – *P. l. luschani* (0.017342), Kemer – *P. luschani birandi* (0.011782), and Patara – *P. l. luschani* (0.009074) populations (Table 2). The number of segregating sites amongst the haplotypes of a population varied between 1 and 55 bp. The highest numbers were observed in the Demre, Patara, and Kalkan populations of *P. l. luschani*. Numbers were also high in Kemer – *P. luschani birandi* – and İzmir – *P. ledereri*. Pairwise differences between intrapopulation haplotypes ranged from 0.13 to 19.23, in parallel with the number of segregating sites. The highest scores were determined in the Demre (19.23), Kemer (13.07), Patara (10.06), and Kalkan (7.08) populations, with the second belonging to *P. luschani birandi* and the other three to *P. l. luschani*. These values were lower than 5.00 in all other populations, with the lowest values

Table 2. Genetic diversity indices of 18 populations of the *Poecilimon luschani* species group. Shown from left to right are: number of unique haplotypes (*k*, with indels for 16S rDNA), number of unique haplotypes of 16S rDNA excluding indels (*K*), haplotype diversity ($h \pm SD$), number of segregating sites (*s*), nucleotide diversity ($\pi \pm SD$) and mean pairwise differences between *k* haplotypes ($d \pm SD$) were estimated for cytochrome c oxidase subunit I (COI) sequences

		COI						16S rDNA		
Species	Locality	*N*	*k*	*h*	*s*	π	*d*	*N*	*k*	*K*
Poecilimon ledereri	Turkey: İzmir, Bozdağ Mountain	24	13	0.9457 ± 0.0223	19	0.003345 ± 0.001952	3.710145 ± 1.941777	18	4	4
Poecilimon orbelicus	Bulgaria: Pirin Mountain	15	2	0.1333 ± 0.1123	1	0.000120 ± 0.000212	0.133333 ± 0.209858	17	3	4
Poecilimon tuncayi	Turkey: Aydın, Çine	17	9	0.9118 ± 0.0424	15	0.003328 ± 0.001980	3.691176 ± 1.962517	34	3	7
Poecilimon egrigozi	Turkey: Kütahya, Eğrigöz Mountain	18	12	0.9216 ± 0.0466	14	0.004096 ± 0.002362	4.542484 ± 2.342492	2[α]	2	2
Poecilimon helleri	Turkey: Balıkesir, Kazdağı Mountain	16	4	0.6917 ± 0.0736	11	0.001706 ± 0.001150	1.891667 ± 1.138349	12	1	2
Poecilimon luschani chobanovi	Turkey: Antalya, Eşen Çayı 1	13	2	0.2821 ± 0.1417	1	0.000254 ± 0.000326	0.282051 ± 0.321561	18	1	1
	Turkey: Antalya, Eşen Çayı 2	14	10	0.9560 ± 0.0377	10	0.002339 ± 0.001494	2.593407 ± 1.476400	14	3	5
	Turkey: Antalya, Eşen Çayı 3	10	7	0.9333 ± 0.0620	13	0.004228 ± 0.002558	4.688889 ± 2.507921	14	4	5
	Turkey: Antalya, Akdağ Mountain	–	–	–	–	–	–	6	1	1
	Turkey: Antalya, Erentepe Mountain	–	–	–	–	–	–	9	2	3
Poecilimon luschani luschani	Turkey: Antalya, Demre	24	11	0.8841 ± 0.0399	55	0.017342 ± 0.008870	19.231884 ± 8.823532	15	4	6
	Turkey: Antalya, Kalkan	14	6	0.7473 ± 0.1114	27	0.006381 ± 0.003578	7.076923 ± 3.535106	16	4	4
	Turkey: Antalya, Patara	20	11	0.8947 ± 0.0520	26	0.009074 ± 0.004836	10.063158 ± 4.801537	15	3	4
Poecilimon luschani birandi	Turkey: Antalya, Termessos	9	2	0.5000 ± 0.1283	1	0.000451 ± 0.000477	0.500000 ± 0.466504	19	3	3
	Turkey: Antalya, Bakırlıdağ Mountain	15	2	0.1333 ± 0.1123	17	0.002044 ± 0.001333	2.266667 ± 1.318248	14	3	7
	Turkey: Antalya, Kemer	6	4	0.8667 ± 0.1291	22	0.011782 ± 0.007158	13.066667 ± 6.874429	11	3	4
	Turkey: Antalya, Tahtalıdağ Mountain	–	–	–	–	–	–	4	3	3
	Turkey: Antalya, Olympos	19	9	0.7719 ± 0.0944	17	0.002668 ± 0.001632	2.959064 ± 1.619380	13	3	4
Total		234	104					251	50	69
Outgroups										
Poecilimon obtusicercus	Turkey: Denizli, Tavas	1						1		
P. obtusicercus	Turkey: Antalya, Korkuteli	1						1		
Poecilimon cervus	Turkey: Bolu Yedigöller	1						1		
Poecilimon izmirensis	Turkey: Kütahya, Egrigöz Mountain	1						1		

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found in the Balkan – *P. orbelicus* (0.13), Eşen1 – *P. luschani chobanovi* (0.28), and Termessos – *P. luschani birandi* (0.50) populations.

As no species shared haplotypes, fixation indices were calculated only for 11 populations belonging to the *P. luschani* complex (Table 3). The analysis of pairwise differences between populations corrected by the TamuraNei + Gamma (G = 1.061) model showed that F_{ST} ranged from 0.01575 to 0.99299, indicating significant genetic structuring of the *P. luschani* complex. Of the total of 45 pairs, the pairwise differences of 43 were statistically significant ($P < 0.01$ for two and $P < 0.001$ for the others), and nonsignificant for the remaining two (Bakırlıdağ–Termessos for *P. luschani birandi* and Kalkan–Patara for *P. l. luschani*). Low and nonsignificant F_{ST} values between these populations indicate either a high rate of gene flow or recent splitting (Table 3).

Table 3. Pairwise F_{ST} values (below diagonal) and their *P*-values (above diagonal) for ten populations of *Poecilimon luschani.* TamuraNei + Gamma (1.061), number of permutations = 10 000; F_{ST} values lower than 0.5 are in bold

	Poecilimon luschani birandi				*Poecilimon luschani luschani*			*Poecilimon luschani chobanovi*		
	Termessos	Bakırlıdağ	Kemer	Olympos	Demre	Kalkan	Patara	Eşen1	Eşen2	Eşen3
Termessos		0.40491	0.00030	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Bakırlıdağ	**0.02476**		0.00277	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Kemer	0.51265	**0.47874**		0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00010
Olympos	0.89819	0.86807	0.66631		0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Demre	0.63456	0.65443	0.51532	0.67610		0.00000	0.00000	0.00000	0.00000	0.00000
Kalkan	0.90852	0.90658	0.82225	0.90895	**0.49638**		0.25255	0.00000	0.00000	0.00000
Patara	0.85900	0.86552	0.78665	0.87222	**0.48528**	**0.01575**		0.00000	0.00000	0.00000
Eşen1	0.99299	0.97348	0.91714	0.96424	0.57795	0.75865	0.63400		0.00000	0.00000
Eşen2	0.96392	0.95033	0.88682	0.94453	0.52875	0.65726	0.53491	0.83078		0.00000
Eşen3	0.94809	0.93800	0.85285	0.93412	0.50481	0.67466	0.57801	0.86521	0.75847	

Time estimation

The ILD test suggested a similar evolutionary pattern ($P = 0.167$) for the sequences of the 16S and COI markers and therefore both were concatenated into a single matrix. The total length of the concatenated sequences was 1401 bp. Of these, 1011 sites were invariable, 390 were variable, and 326 of the variable sites were parsimony informative. Seventy-three different haplotypes were defined from the concatenated matrix. Of these, four belonged to outgroups (two to *Poecilimon obtusicercus*, and one to each of *Poecilimon cervus* and *Poecilimon izmirensis*) and 69 to the ingroup taxa.

The null hypothesis of an equal evolutionary rate throughout the tree calculated from the concatenated data matrix was rejected at the 5% significance level (log likelihood = 6508.467, $P < 0.001$) in the MLC analysis. The results of the whole-tree statistics computed in SymmeTREE using the ML, MP, and BIP tree topologies given in Boztepe *et al*. (2013) confirmed the presence of both tree imbalance and significant variation in diversification rates within *P. luschani* at an intermediate level (Table 4). Although the Slowinski–Guyer and Δ_1 and Δ_2 likelihood rate shift statistics did not distinguished any node, this may be because of the polytomic Nodes 3 and 8 (see Fig. 2).

As MLC and SymmeTREE suggested nonclock evolution for the data set, the BEAST analysis was run under two different clock models: the uncorrelated lognormal relaxed clock (ULRC) and random local clock (RCM). MODELTEST applied to the concatenated data matrix suggested a general time-reversible model (GTR) with gamma correction (G = 1.2528) and invariable sites (I = 0.6110) according to Akaike's information criterion. The BEAST analysis, under a GTR + I + G model, supported nonclock-like evolution of this segment of DNA [ucld.stdev = 1.089; 95% highest posterior density

Table 4. Tests of amongst-clade diversification rate using four topology-based indices of whole-tree symmetry in the maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference phylogeny (BIP) trees of the *Poecilimon luschani* group given in Boztepe *et al*. (2013). Values represent the test statistics with the *P*-value in parentheses for each tree. The range of values represents the upper and lower bounds generated when the analyses were repeated with 1 000 000 random resolutions of polytomies with different degrees of symmetry. Indices are the Colless Index (IC), Shao & Sokal Index (B1), and the *M* statistics M_π and M_Σ ($P < 0.05$ are indicated in bold)

Trees	IC	M_π	M_Σ	B1
	Min (0.025)	Min (0.025)	Min (0.025)	Min (0.025)
	Max (0.975)	Max (0.975)	Max (0.975)	Max (0.975)
ML	449 (**0.00137**)	7.40E-13 (**0.02088**)	55.4465 (**0.02**)	37.4206 (0.1574)
	328 (0.05772)	4.23E-09 (0.46562)	60.3291 (0.58969)	41.3516 (0.94473)
MP	406 (**0.00584**)	4.36E-12 (**0.04833**)	55.966 (**0.03367**)	37.6182 (0.19082)
	275 (0.20608)	4.05E-08 (0.6906)	60.9881 (0.71011)	41.4849 (0.95515)
BIP	447 (**0.0018**)	2.28E-12 (**0.03528**)	56.1093 (**0.03901**)	37.7349 (0.20887)
	326 (0.06038)	9.67E-09 (0.55002)	60.8853 (0.6942)	41.1968 (0.93084)

(HPD): 0.771–1.391, ESS: 1302]. The BFs of the clock models suggested that the ULRC model ($\log_{10}$ BF = –6590.586) is more suitable for our data set than RCM ($\log_{10}$ BF = –6610.944).

The BEAST tree differs from the combined phylogenetic tree (given in Boztepe *et al*., 2013) in one node. The node including three branches as *P. tuncayi* + (*P. egrigozi* + *P. helleri*) + *P. luschani* on the combined tree occurs as (*P. tuncayi* + (*P. egrigozi* + *P. helleri*)) + *P. luschani* on the BEAST tree. BEAST (Fig. 2) estimated the TMRCA for the *P. luschani* species group as 1.323 Myr, corresponding to the Calabrian stage in the Pleistocene. *Poecilimon ledereri* branches off basally and the remaining species constitute a sister phylogroup that shared a MRCA 1.075 Myr BP, also in the Calabrian. At the next step on the tree, the Balkan species *P. orbelicus* diverges from the remaining Anatolian lineage of four species. BEAST suggested the TMRCA for this four-species Anatolian lineage to be 1.007 Myr, in the Calabrian (BEAST suggested no HPD interval as the posterior probability for this node was lower than 0.50). The Anatolian lineage consists of two infralineages, the first including *P. tuncayi* + (*P. helleri* + *P. egrigozi*) and the second the *P. luschani* complex. The TMRCA for the first clade is 0.852 Myr (BEAST suggested no HPD interval as the posterior probability for this node was lower than 0.50) and that for the second is 0.839 Myr, both corresponding to the transition from the Middle to the Late Pleistocene. Within the first clade, *P. helleri* and *P. egrigozi* share a MRCA 0.574 Myr (see Table 5 for HPDs of the nodes).

The *P. luschani* complex includes two phylogroups (classified as three subspecies; Fig. 2). The first phylogroup consists of haplotypes from the eastern part of the species' range (Termessos, Bakırlıdağ, Kemer, and Olympos populations; eastern phylogroup, EP hereafter) in addition to six haplotypes of the Demre population. BEAST suggested the TMRCA for this phylogroup to be 0.652 Myr. The haplotypes of the EP other than those from the Demre population shared a MRCA 0.440 Myr. The phylogroup sister to the EP includes haplotypes from the west and south populations (Kalkan, Patara, Eşen1, Eşen2, and Eşen3; western phylogroup, WP hereafter) in addition to four haplotypes from the Demre population. The BEAST chronogram suggested the TMRCA to be 0.625 Myr for the WP. BEAST suggested intraspecies radiation of the *P. luschani* complex during the intense glacial period of the Late Pleistocene (see Table 5 for HPDs of the nodes).

Demographic analyses

The results of Tajima's *D*, Fu's *Fs*, Fu and Li's *D**, Ramos–Onsins and Rozas's R_2, and the *g* parameter tests are presented in Table 5. Of these, the negative *g* parameter suggested a historical contraction in three populations of *P. l. luschani*: Demre, Kalkan, and Patara (Fig. 3G–I). Fu's *Fs* also supported this contraction, strongly for Demre and weakly for Kalkan. Consistent with Fu's *Fs* and the negative *g* parameters, the GMRF skyride plots indicated sudden reductions that started around 300 and 120 Kyr BP for Demre and Patara, respectively, but a weak reduction for Kalkan (Fig. 3G–I). In contrast to the above-defined reduction in population size, the multimodal shape of the mismatch distribution in these three populations (Fig. 4G–I) indicates a structured population size or gene admixture. The values of Fu's *Fs* and *g* parameters estimated a significant historical expansion for Eşen2 – *P. luschani chobanovi*. This expansion is supported by the skyride plot (Fig. 3E) and by the unimodal mismatch distribution (Fig. 4E). Fu and Li's *D** suggested a weak expansion for two populations of *P. l.*

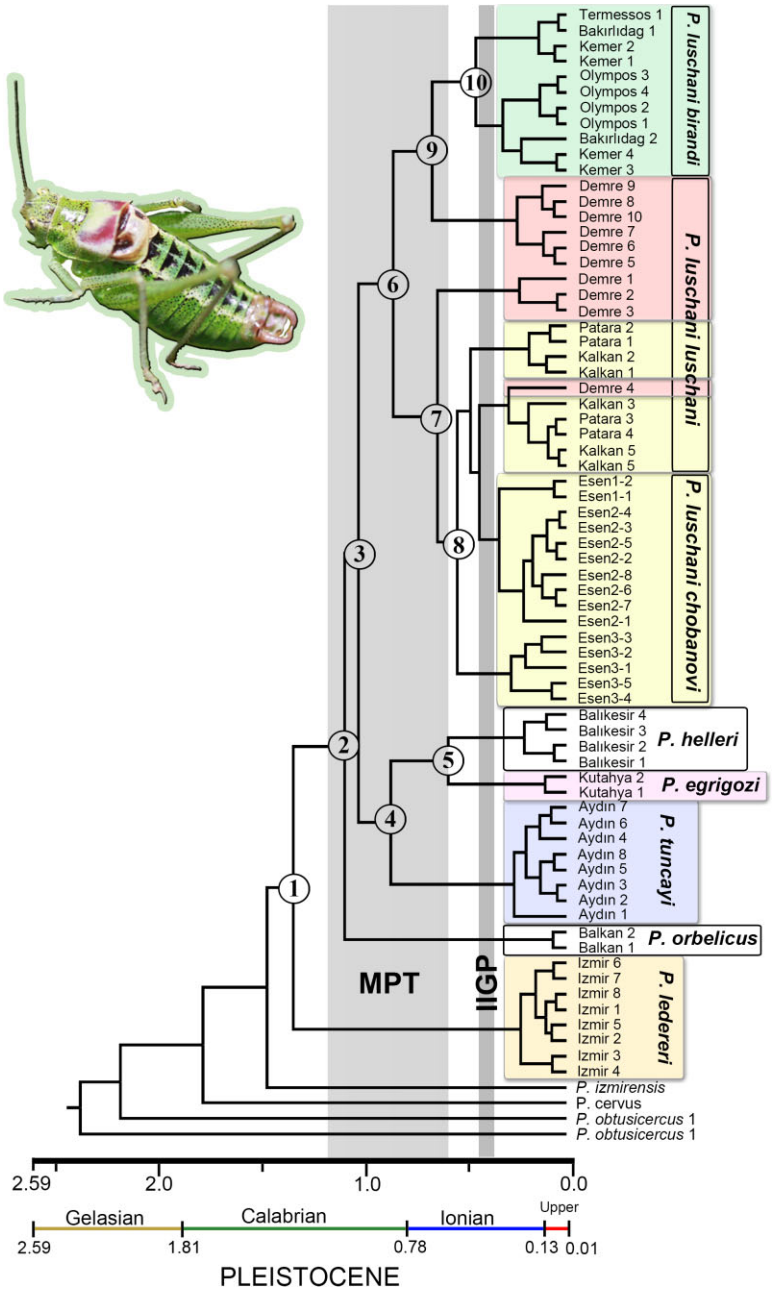

Figure 2. BEAST chronogram based on concatenated sequences of cytochrome c oxidase subunit I + 16S rDNA for the *Poecilimon luschani* species group. Divergence times inferred according to Bayesian inference with an uncorrelated log-normal relaxed clock in BEAST (MPT, Mid-Pleistocene Transition; IIGP, initiation of intense glaciation periods; for details of populations see Table 1 and for the haplotype codes see Tables S1, S2).

birandi (Olympos and Bakırlıdağ; Fig. 3J, K). However, the multimodal shape of the mismatch distribution in these two populations suggests a stable population history (Fig. 4J, K). However, as the skyride plots of Bakırlıdağ and Olympos show a sudden reduction that started around 250–200 Kya (Fig. 3J, K), these negative values of Fu and Li's D^* indicate an expansion following a bottleneck for these two populations. This is also supported by Tajima's D for the Bakırlıdağ population. The g parameter for Aydın – *P. tuncayi* indicates a historical expansion. The unimodal shape of the mismatch distribution (Fig. 4B), consistently the nonsignificant low values of SSD and *Hri*, support this expansion. Moreover, the skyride plot of this population shows several fluctuations during the last 150 Kyr (Fig. 3B). Different demographic estimators are in con-

Table 5. The median and 95% highest posterior density (HPD) credibility intervals for the nodes numbered on the BEAST chronogram given in Figure 2

Node number	Time to most recent common ancestor for	Mean	HPD
1*	*Poecilimon luschani* group	1.323	0.89–1.75
2*	*Poecilimon orbelicus* + ((*Poecilimon tuncayi* + (*Poecilimon egrigozi* + *Poecilimon helleri*)) + *P. luschani*)	1.075	0.74–1.40
3	*P. luschani* + (*P. tuncayi* + (*P. egrigozi* + *P. helleri*))	1.007	†
4	*P. tuncayi* + (*P. egrigozi* + *P. helleri*)	0.852	†
5*	*P. egrigozi* + *P. helleri*	0.574	0.25–0.89
6*	*Poecilimon luschani birandi* + (*Poecilimon luschani luschani* + *Poecilimon luschani chobanovi*)	0.839	0.58–1.13
7*	*P. l. luschani* – Demre+ (*P. l. luschani* – Demre, Patara, Kalkan+ *P. l. chobanovi*)	0.625	0.42–0.85
8*	*P. l. chobanovi*- Eşen3 + (*P. l. luschani* – Demre, Patara, Kalkan+ *P. l. chobanovi*- Eşen1, Eşen2)	0.531	0.34–0.71
9*	*P. l. birandi* + *P. l. luschani* – Demre	0.652	0.42–0.94
10*	*P. l. birandi* – Termossos, Bakırlıdağ, Kemer + *P. l. birandi* – Olympos, Bakırlıdağ, Kemer	0.440	0.25–0.66

*nodes constrained in the BEAST analyses.
†BEAST suggested no HPD.

flict for the history of İzmir – *P. ledereri*. Fu's *Fs*, *g* parameter, and the unimodal mismatch distribution suggest a weak expansion. However, the skyride plot of this population (Fig. 3A) indicates a gradual reduction through the last 200 Kyr. Of the different demographic estimators, only Tajima's *D* suggested a weak expansion for Balıkesir – *P. helleri*, whereas the others suggest a stable history. The demographic parameters, mismatch distribution, and skyride plots suggest no significant departure in population size for Kütahya – *P. egrigozi* (Table 5, Figs 3C, D, 4C, D).

DISCUSSION

Radiation history of the group and corresponding historical events

The BEAST chronogram shows that the *P. luschani* species group shared a common ancestor 1.323 Myr BP. Descending from the group's ancestral node, there are four nodes (Nodes 2–5) corresponding to speciation events on the BEAST tree in addition to five internal nodes (Nodes 6–10) in the *P. luschani* complex (Fig. 2; Table 5). Nodes 2 and 3 are almost synchronous around 1.1–1.0 Myr BP. Nodes 4 and 6 are 0.85–0.80 Myr and Node 5, the common ancestor of *P. helleri* and *P. egrigozi*, is 0.574 Myr old. Ages of the internal nodes of the *P. luschani* complex vary from 0.652 to 0.44 Myr for the main infragroups. Thus, according to the nodes' ages there are five radiation steps in the species group at: (1) 1.323, (2) 1.1–1.0, (3) 0.85–0.84, (4) 0.65–0.53, and (5) 0.44 Myr BP. Of these, the second and third radiation times fall within the Mid-Pleistocene Transition (MPT), a period characterized by lengthening of the glacial periods from 41- to 100-Kyr cycles (Meyers & Hinnov, 2010). The fourth radiation time corresponds to the end of the MPT and the fifth to the beginning of the intense glaciations periods (Lisiecki & Raymo, 2007; Meyers & Hinnov, 2010; Cohen, Finney & Gibbard, 2012; Ivashchenko *et al*., 2013). Additionally, when the means of the TMRCAs of the ten nodes indicated on the BEAST chronogram were plotted on a time chart of Pleistocene temperature cycles, seven corresponded to warm and three to cold periods (Fig. 5). Thus, when the means of the TMRCAs are considered, it can be assumed that most of the taxa arose from ancestral populations that were isolated during a warm period. Although the HPDs of the nodes weaken this assumption, we suggest that cold periods provided an opportunity to colonize new range areas and to found new populations, whereas warm periods caused fragmentation and divergence of these populations via isolation.

The good correlation of the time estimations from BEAST with the transitions in the climatic system or with severe fluctuations in climate parameters suggests that climate shifts in the Pleistocene are the main driver of divergence and speciation. The radiation period of the *P. luschani* group constitutes a rare example of the climatic oscillations in the recent half of Pleistocene leading to speciation (Hampe & Petit, 2005). This temporal pattern of radiation is in conflict with several other Anatolian lineages. The tettigonnid genus *Anterastes* is one such example. It prefers a cold climate, is present in high altitude habitats, and radiated in Anatolia in the western half of the Anatolian Diagonal. All of the 14 cladogenetic events that resulted in species took place

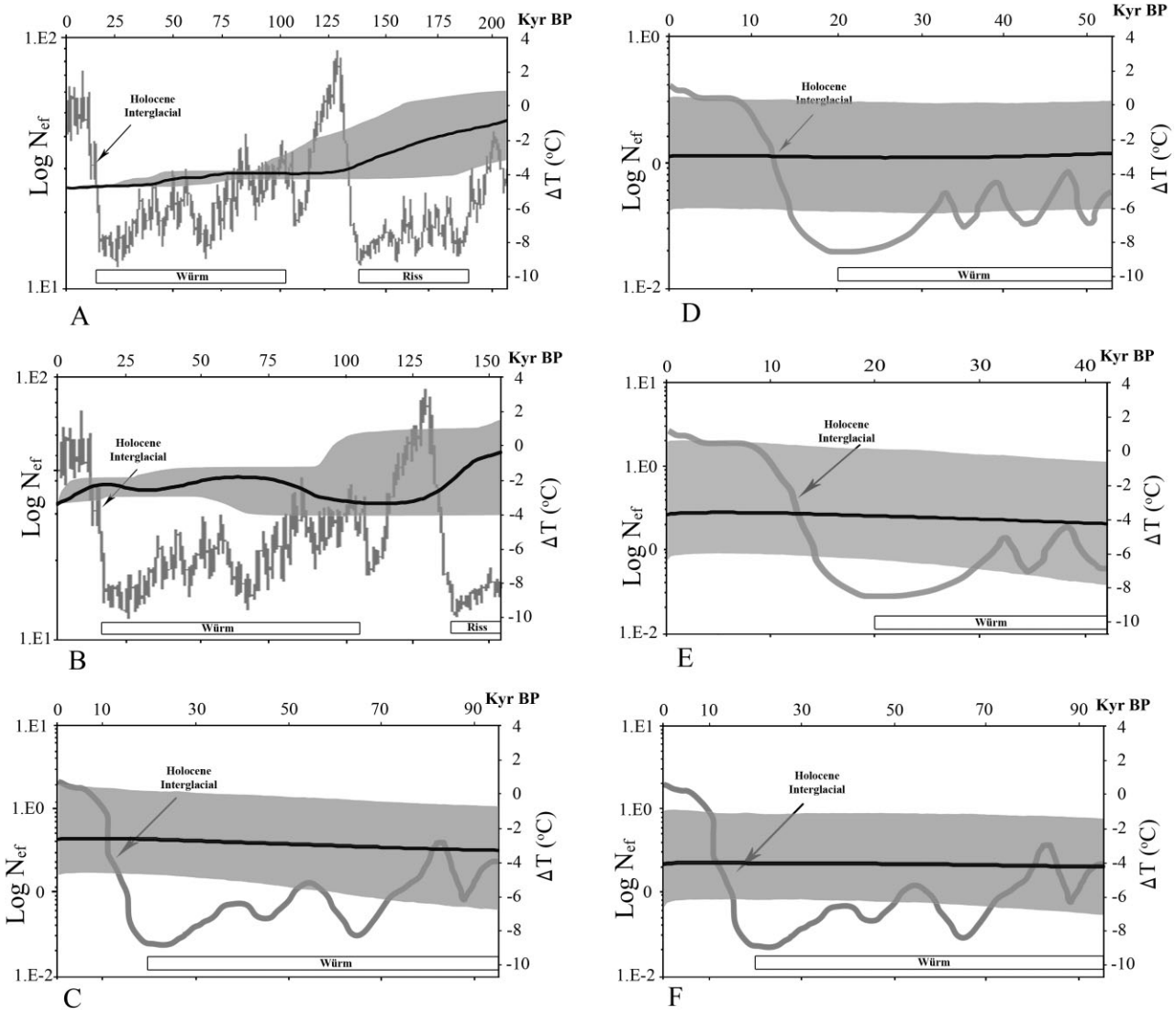

Figure 3. Gaussian Markov random field skyride plots for 11 populations of the *Poecilimon luschani* species group. The median estimates are shown as a thick solid line, and the 95% highest posterior density limits are shown by the grey area. The background grey graphs show fluctuations in temperature during the respective periods of the Pleistocene. The upper and lower margins show the time in kiloyears (Kyr), the left margin shows the log of the effective population size (N_{ef}), and the right margin the departure in temperature (ΔT in °C). Names of the glacial periods are indicated in the boxes at the bottom of the graphs. The background graph shows the temperature reconstruction of Vostok; modified from http://agwobserver.wordpress.com. A, İzmir (*P. ledereri*); B, Aydın (*P. tuncayi*); C, Kütahya (*P. egrigozi*); D, Balıkesir (*P. helleri*); E- Eşen2 (*P. l. chobanovi*); F, Eşen3 (*P. l. chobanovi*); G, Demre (*P. l. luschani*); H, Kalkan (*P. l. luschani*); I, Patara (*P. l. luschani*); J, Olympos (*P. l. birandi*); K, Bakırlıdag (*P. l. birandi*).

prior to 1.2 Myr BP, most of them in the Pliocene or Early Pleistocene (Çıplak *et al*., 2010). Similarly, *Troglophilus* (Kaya *et al*., 2012), *Spermaphilus* (Gündüz *et al*., 2007), *Arabis* (Ansell *et al*., 2011), *Hyla* (Gvoždik *et al*., 2010), *Mertensiella caucasica* (Tarkhnishvili *et al*., 2000), and *Rana* (Veith *et al*., 2003; Akın *et al*., 2010) are some other examples from Anatolia of species-level radiations that occurred prior to the Middle Pleistocene. Although it is suggested that the present biodiversity mainly originated in the Pliocene (Hampe & Petit, 2005), some recent examples, such as the *P. luschani* species group, indicate that climatic oscillations are machines of speciation (Voje *et al*., 2009).

Of the eight taxa, *P. orbelicus* is the only representative of the group in the Balkans. The most basal branch of the species group is in Anatolia and the Balkan species shares the most recent common ancestor with its Anatolian relatives 1.075 Myr. These findings indicate that the Balkan

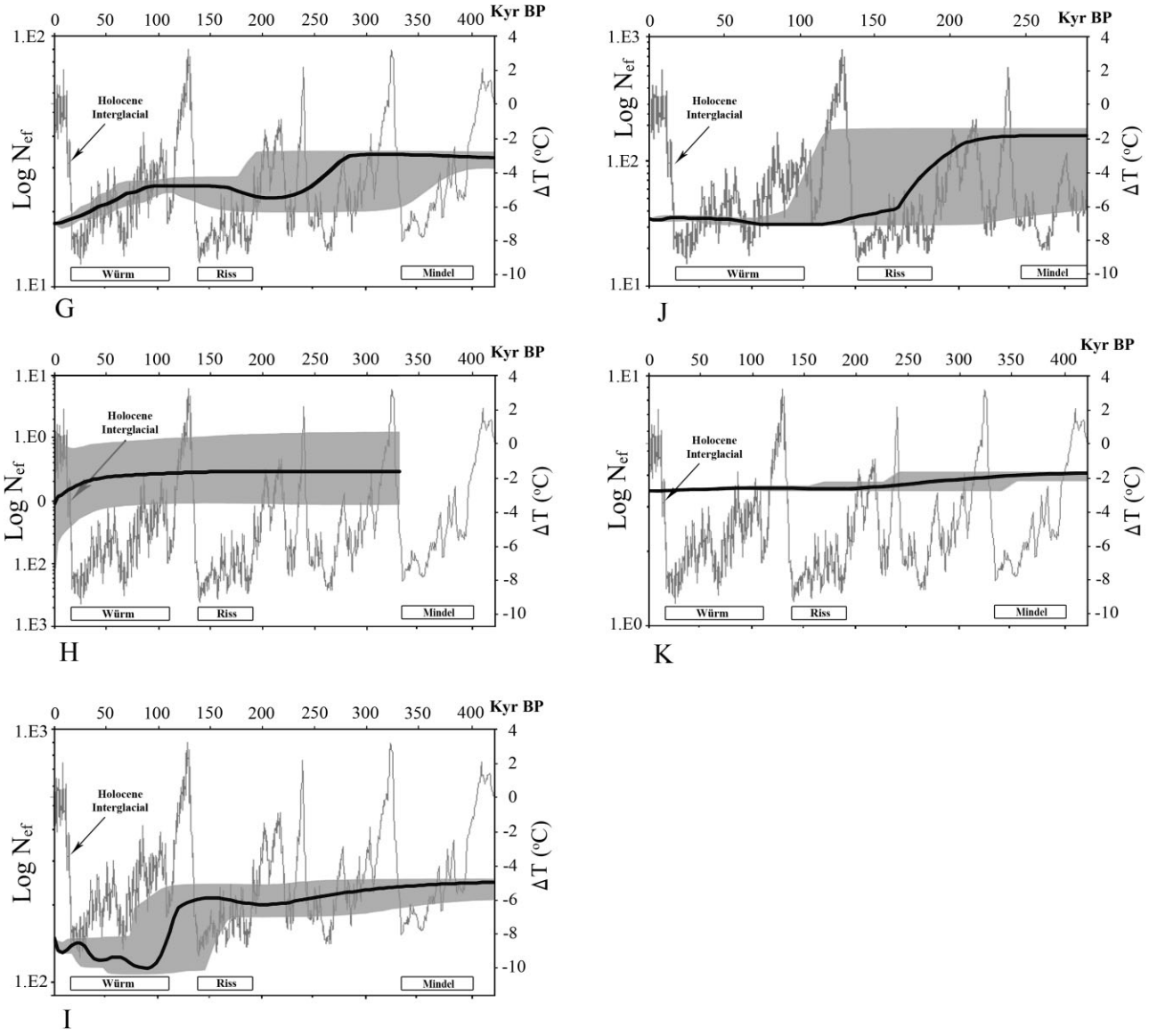

Figure 3. *Continued.*

species was founded by an ancestral stock that dispersed from Anatolia to the Balkans around 1.075 Myr and diverged afterwards. There are two possible corridors for this dispersal, one through Dardanelles and the second through Bosporus. There are no representative of this species group around the İstanbul and Kocali Peninsulas, which border Bosporus. Additionally, the present waterway of Bosporus is about 5000 years old (Gökaşan *et al*., 2010), but there was a water strait connecting the Marmara Sea to the Black Sea through Sapanca Lake and the Sakarya River up to 800 Kyr BP (Elmas, 2003). By contrast, there was a terrestrial corridor between Anatolia and Thrace through Dardanelles up to 150 Kyr BP (Elmas, 2003). In addition, the range of *P. helleri* is very close to Dardanelles. All these factors support the assumption that dispersal to the Balkans occurred through Dardanelles, as suggested for the dispersal of *Anterastes serbicus* to the Balkans (Çıplak *et al*., 2010).

From the distribution and speciation patterns, the most plausible scenario for the phylogeographical history of this species group can be outlined as follows. Ancestral stock was present in Aegean Anatolia around/prior to 1.3 Myr BP. This ancestral stock dispersed to a large part of Anatolia and the Balkans to colonize the present total range of the species group after this date, but prior to 1.0 Myr BP, around the MPT. The synchronous radiation of the Balkan species *P. orbelicus* and the four-species Anatolian crown clade supports this assumption. The later radiation steps within the Anatolian crown group occurred locally, during the

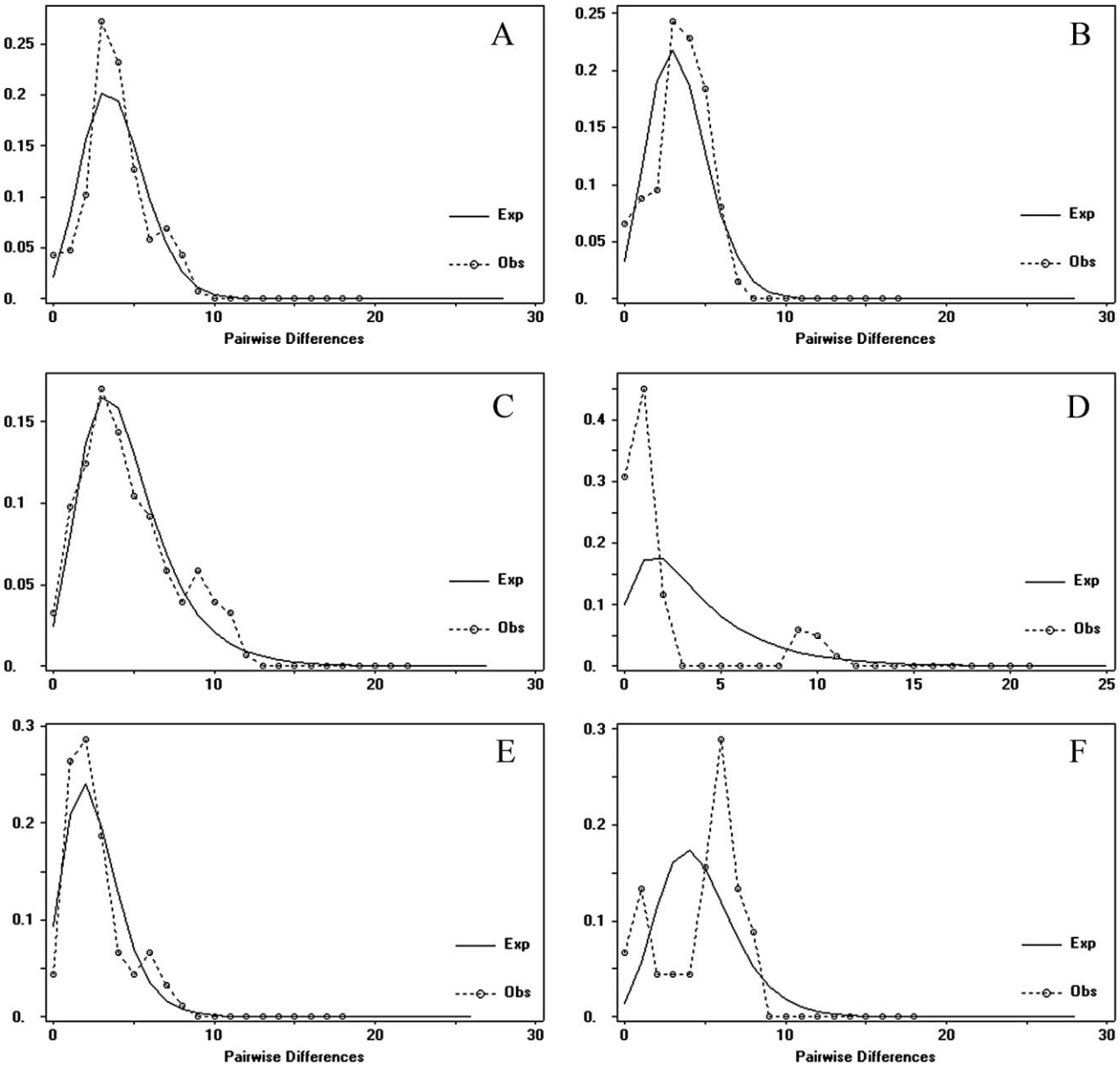

Figure 4. Mismatch distributions for 11 populations of the *Poecilimon luschani* species group for cytochrome c oxidase subunit I (COI) sequences. The continuous and interrupted (connecting circles) lines indicate the expected (Exp) and observed (Obs) distributions of pairwise differences obtained by fitting a model of sudden population expansion. A, İzmir (*P. ledereri*); B, Aydın (*P. tuncayi*); C, Kütahya (*P. egrigozi*); D, Balıkesir (*P. helleri*); E- Eşen2 (*P. l. chobanovi*); F, Eşen3 (*P. l. chobanovi*); G, Demre (*P. l. luschani*); H, Kalkan (*P. l. luschani*); I, Patara (*P. l. luschani*); J, Olympos (*P. l. birandi*); K, Bakırlıdag (*P. l. birandi*).

transition to lengthened glacial periods around 800 Kyr BP. The sister species *P. helleri* and *P. egrigozi* occur in high-altitude habitats in north-western Anatolia, on Kazdağ Mountain and Eğrigöz Mountain, respectively. As there are no records in the lowlands of Thrace and northern Anatolia, these two species were possibly founded by some ancestral stock that remained on these summits when suitable habitats disappeared during an arid/warm period around 600 Kyr BP. These ancestral populations possibly managed to survive later climatic cycles by local vertical range shifts, as suggested for some other mountainous Anatolian lineages (Çıplak *et al*., 2010). *Poecilimon tuncayi* is another species restricted to Aegean Anatolia. Its range is side by side with that of *P. ledereri*. Although an overlap in distribution is possible, they have not been found

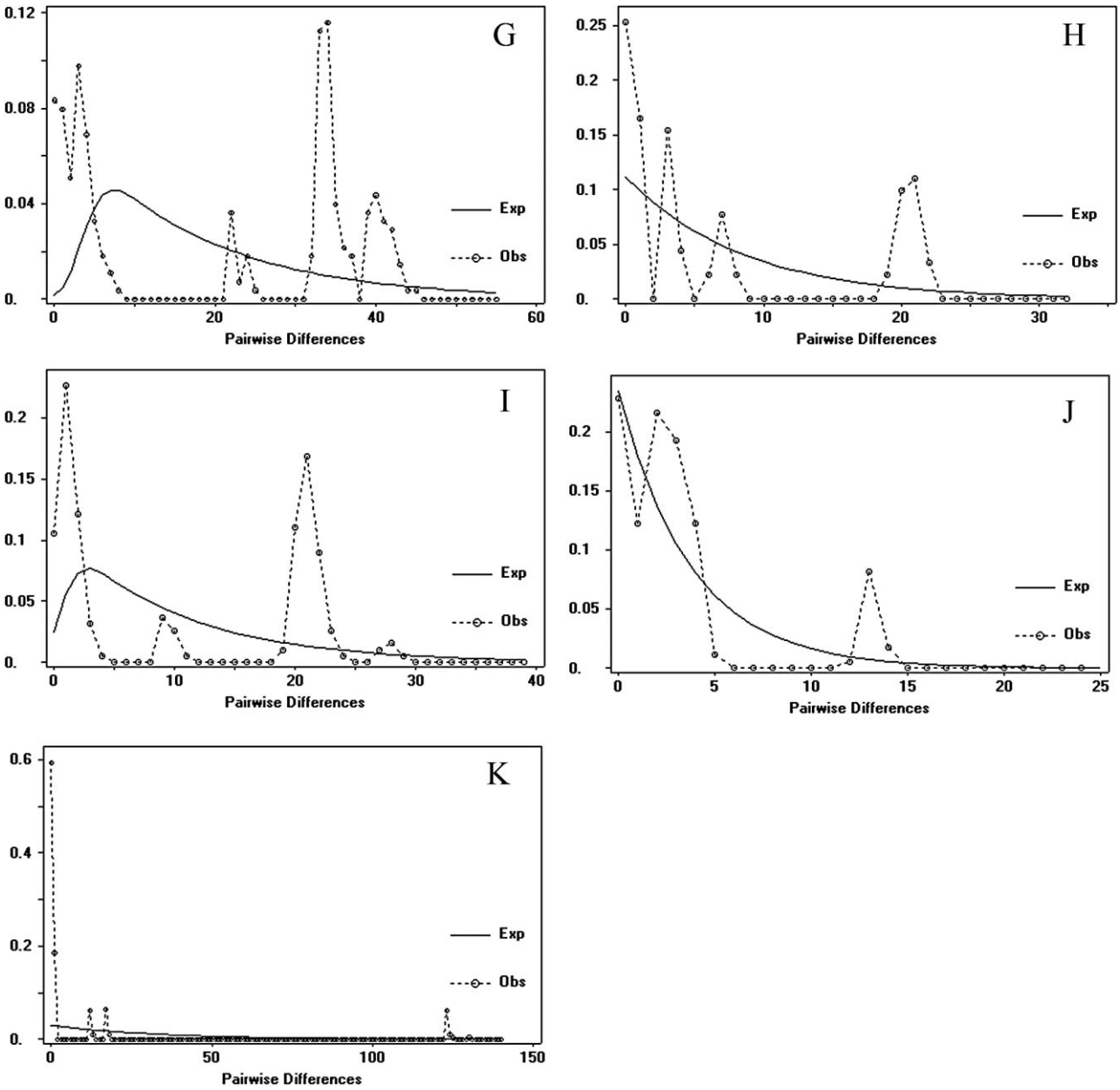

Figure 4. *Continued.*

sympatrically; Menderes River seems to constitute a border and barrier for these two species. As there was a larger water body along the Menderes Valley from the Aegean Sea to Bafa Lake in the Late Pleistocene (Ergin *et al*., 2007), it was possibly also the main barrier in the past that prevented mixing of *P. ledereri* and *P. tuncayi* and directed their divergence.

The radiation of the crown clade *P. luschani* complex in the south-west corner of Anatolia was evaluated in detail by Kaya *et al*. (2012) using 16S rDNA data. The present data, based on two marker genes, add significant new findings to these previous data. The first is that the time estimation in the present study brings the TMRCAs for the entire and the internal groups of the *P. luschani* complex to a more recent time than that suggested by Kaya *et al*. (2012). The time estimation in the present study seems more plausible than that of Kaya *et al*. for two reasons. First, the presence of shifts in divergence rate in the history of the lineage as suggested by SymmeTREE, and the nonclock-like evolution pattern of the current data set, may be the reasons for this conflict. Second, the present data set includes the concatenated sequences of two markers, whereas Kaya *et al*. (2012) used a single marker. Thus,

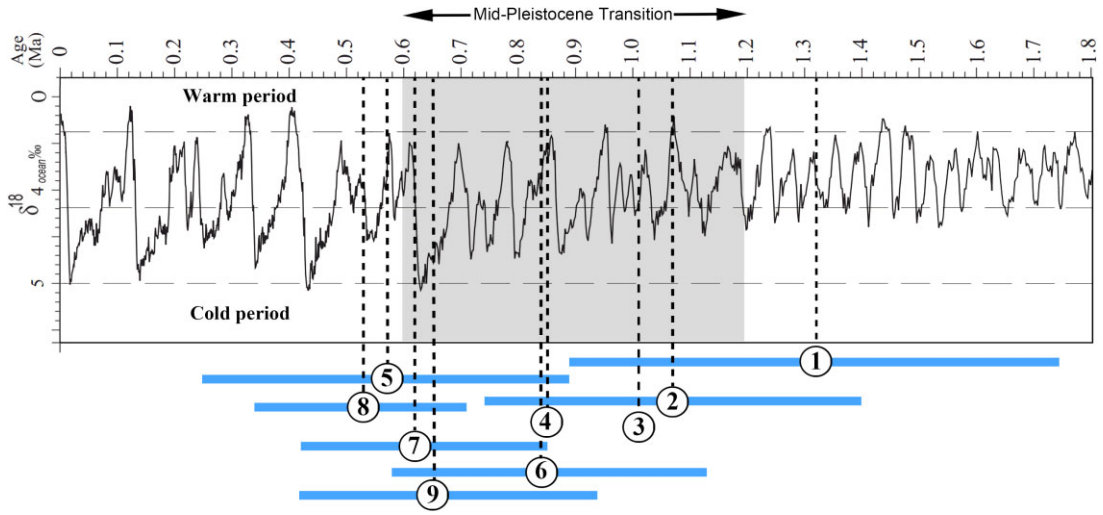

Figure 5. Climatic cycles during the Mid and Late Pleistocene and time to the most recent common ancestor of the main nodes in the *Poecilimon luschani* species group. The median estimates are shown by a dashed line; the 95% highest posterior density (HPD) limits are shown by the pale blue horizontal bars. Node numbers, median estimates, and HPD are shown in Table 4 (time chart for climatic cycles modified from Cohen *et al*., 2012). (Colour version of figure available online.)

the present, larger data set may be considered as more reliable. Another new finding is the more robust phylogeny, which suggests the Demre population to be the ancestral stock and the eastern and western phylogroups to be later derived lineages. Apart from these findings, the newly added data support the statements in Kaya *et al*. (2012) that the geographical evolution of the local area (Öner, 2000) was the main driver producing the present level of diversity. A Pleistocene lake and associated streams that separated the eastern and western phylogroups seems to be the most plausible barrier that led to the differentiation of local forms (further details are given below).

Ecological preferences, environmental changes, and historical demography

Representatives of the species group have been recorded at different altitudes from sea level up to 2300 m elevation in open habitats or forest clearings (Çıplak *et al*., 2008; Kaya *et al*., 2012; Boztepe *et al*., 2013). However, there are some species or single populations confined to particular altitudes. Accepting presence at high altitudes as one character state and in lowlands as the reverse state, the most basal branch *P. ledereri* can be assumed to be polymorphic as it occurs over a relatively wide altitudinal range on slopes of the Bozdağ and Aydın mountain ranges. *Poecilimon tuncayi* is the only species confined to the lowlands in Aegean Anatolia, in the Aydın and Muğla provinces of Turkey. The species present in the northern part of the group's range are confined to alpine or subalpine open grassland habitats above treelines: *P. orbelicus* on the Rhodopian Mountains, *P. helleri* on Kazdağ Mountain, and *P. egrigozi* on Egrigozi Mountain. The *P. luschani* complex is distributed over a wide altitudinal range from sea level up to 2300 m and exhibits significant altitudinal size clines that follow the converse Bergmann rule (Çıplak *et al*., 2008). Of the three subspecies, all populations of *P. l. luschani* occur only at low altitudes whereas *P. luschani birandi* and *P. luschani chobanovi* have populations over a wide altitudinal range from sea level up to 2300 m. Evaluating habitat preferences in relation to phylogenetic relationships (see Boztepe *et al*., 2013 and BEAST chronogram given in Fig. 2), altitudinal preference has changed several time within the lineage (Fig. 6). As the radiations of *P. orbelicus*, *P. tuncayi*, *P. egrigozi* + *P. helleri*, and *P. luschani* seem almost synchronous and as the most basal branch, *P. ledereri*, occurs at moderate altitudes, the habitat preferences of *P. helleri* + *P. egrigozi* evolved once from a polymorphic ancestor. The habitat preferences of *P. tuncayi* living in the lowlands and those of *P. orbelicus* in the highlands evolved independently from the same polymorphic ancestor (Fig. 6). The evolution of habitat preferences in subspecies or populations of *P. luschani* is more complex. The BEAST tree suggested that the Demre population of *P. l. luschani* is the ancestral stock. Further, all populations belonging to this subspecies occur at sea level. Thus, it is possible that the ancestral habitat preference of this species was low altitudes. As each of the other two subspecies occurs over a wide altitudinal range, living in highland habitats can be considered as a secondarily evolved state in some populations of the *P. luschani* complex (Fig. 6).

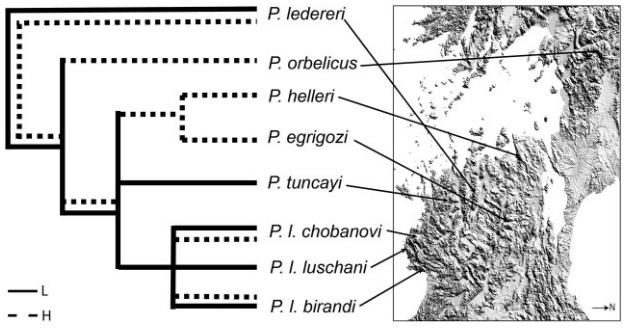

Figure 6. Evolution in ecological preferences of the species, subspecies, and populations of the *Poecilimon luschani* group. Solid lines indicate lowland habitats and dotted lines highland habitats. Polymorphic branches are shown by both lines. Locations of the taxa are indicated on the map given on the right (L, low altitudes; H, high altitudes; polymorphic species indicated by both).

There are some important signals of habitat preference in historical demography. The demographic tests, mismatch distribution, and GMRF skyride plots suggest no or insignificant deviations in population size for the species/populations confined to high mountain habitats, especially those in the northern part of the group's range such as *P. orbelicus*, *P. helleri*, and *P. egrigozi* (Table 6, Fig. 3). The demographic tests and mismatch distribution pattern suggest a weak expansion for the mid-altitude populations such as İzmir – *P. ledereri* and Eşen3 – *P. l. luschani* and some high-altitude populations such as Olympos and Bakırlıdağ for *P. l. birandi*. However, the skyride plots suggest historical population declines for the first and for the last two around 200–150 Kya, a time corresponding to the Riss glacial. The only population that significantly expanded in size is Eşen2, which occurs at moderate altitudes. By contrast, nearly all lowland populations experienced significant deviations in effective population size. This is especially evident for the Demre, Patara, and Kalkan populations of *P. l. luschani*. However, the skyride plots suggest fluctuations in population size during the last 150 Kyr for *P. tuncayi* and a reduction for the Demre and Patara populations around 200 Kya, during the Riss glacial (Fig. 3B). Conflict between different demographic estimators is found for some populations such as İzmir (*P. ledereri*), Aydın (*P. tuncayi*), Olympos (*P. luschani birandi*), Demre, Patara, and Kalkan (*P. l. luschani*), but it is prominent for last three. For example, the multimodal pattern of the mismatch distribution indicates a stable population size, whereas the GMRF skyride plots point to a reduction during the Riss glacial (Fig. 3). A multimodal pattern of the mismatch distribution is considered as a sign of a population in equilibrium or that has experienced genetic admixture (Avise, 2000). The above-defined habitat preferences and historical range patterns indicate the likelihood of past

Table 6. Historical demographic estimates for 11 populations of the *Poecilimon luschani* species group

Species	Population	*N*	Tajima's *D*	Fu's *Fs*	Fu and Li's *D**	SSD	Hri	Tau	Θ_0	Θ_1	R_2	*g*
Poecilimon ledereri	İzmir	24	−0.9824	−4.1915*	0.1688	0.0122	0.0465	3.7500	0.0053	0.8420	0.1219	929.0308
Poecilimon tuncayi	Aydın	17	−0.6473	−1.5861	−0.4120	0.0052	0.0241	4.5703	22.910	0.0000	0.1433	1240.629
Poecilimon egrigozi	Kütahya	18	0.4365	−3.7507*	0.8025	0.0137	0.0376	2.9922	1.9580	27.422	0.1383	740.9067
Poecilimon helleri	Balıkesir	16	−1.6222*	1.2684	−2.2241	0.0250	0.1496	0.9980	0.0000	1000.0	0.1570	−317.7295
Poecilimon luschani chobanovi	Eşen2	14	−0.6824	−5.2818***	−0.6775	0.0102	0.0758	2.2226	0.8070	1000.0	0.1571	2095.876
	Eşen3	10	0.0927	−0.8920	−0.2626	0.0387	0.0765	6.6406	25.242	0.0018	0.1727	643.9951
Poecilimon luschani luschani	Demre	24	1.1910	4.8413	0.9196	0.0504*	0.0499*	34.793	0.0000	29.715	0.1231	−50.47066
	Kalkan	14	−0.7100	2.6736	0.7232	0.0532	0.0931	20.975	0.0000	3.360	0.1453	−95.94174
	Patara	20	1.4483	0.6733	−0.0356	0.0828	0.0612	0.5781	2.2711	1000.0	0.1314	−36.11601
Poecilimon luschani birandi	Olympos	19	−1.4837	−1.9654	−2.4512*	0.0197	0.0480	3.0664	0.0000	2.5440	0.1397	−2.891461
	Bakırlıdağ	26	−2.3764**	1.4816	−4.3596**	0.0095	0.2886	3.0000	0.0000	0.3971	0.1644	−67.09378

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
Hri, raggedness index; SSD, sum of squared differences.

splitting and remixing, possibly frequently in these populations. The presence of heterogeneous topography, climate, and vegetation, and associated altitudinal range shifts may have caused splitting and remixing of populations in the past, resulting in the multimodal mismatch pattern seen in our analyses, but not a population in equilibrium.

In summary, the demographic results show that the effects of the climatic cycles differ depending on the altitudinal and latitudinal locations of the populations. The GMRF skyride plots suggest no strict correlation between departure in population size and warm or cold period (Fig. 3); however, the demographic estimators suggest a stable size for the populations in the north and at high altitudes but significant departures for those in the south and lowlands (Table 5, Fig. 4). Departures from stability in these populations are mostly correlated with cold periods. When this range shift pattern is correlated with present habitat preferences, especially those of the populations located in the northern part of the group's range, such as *P. orbelicus*, *P. helleri*, and *P. egrigozi*, selection pressures in favour of adapting to the cold climate in the northern parts of the group's range and in favour of adapting to the warm conditions in the southern part are more likely.

All of these statements support the assumption that climate change is the main driver directing evolution of the species group. There is further support for this assumption. Members of the group are present over a wide altitudinal range, but temperature preference seems to be similar for all populations. Depending on altitudinal height, populations in lowlands emerge in the early part of the year, in April and May, whereas those in highlands occur in June–August (Çıplak *et al*., 2008; Kaya *et al*., 2012). There are some other features of these animals involved in their evolutionary history. First, as they are flightless, long distance dispersal is very unlikely to have taken place; thus, they survived during climate changes autochthonously. Second, they occur in open areas and forest clearings, so a continuous forest or maquis zone may act as a barrier to their dispersal. Such local barriers may prevent gene flow between closely related populations and thus result in their rapid divergence. As almost all haplotypes are unique at population level (Tables S1, S2) and significantly high pairwise F_{ST} values between populations of the *P. luschani* complex (Table 3) clearly indicate local populations with limited gene flow. Third, as they are small animals, a large population may occur in a small area. Altitudinal heterogeneity provided 'refugial habitats' (Kerdelhué *et al*., 2012), allowing them to survive via vertical range changes in a restricted local area (Çıplak, 2008; Çıplak *et al*., 2010). A similar diversification pattern in the present range of the *P. luschani* complex was defined for *Lyciasalamandra* (Veith *et al*., 2008). Thus, altitudinal heterogeneity plays a buffer role for such species to cope with climate shifts. The species in the northern and central parts of the group's range are confined to open habitats on the tops of single mountains. However, altitudinal heterogeneity in the southern part of the range is very prominent. Thus, the taxonomic and genetic diversity patterns of the species group correlate well with the differences in topography and vegetation within the group's range.

CONCLUSIONS

The present data provide an opportunity to infer some general conclusions. First, the radiation history of the *P. luschani* group constitutes one of the rare examples of changes in the climate system during the Pleistocene working as a speciation driver. All of the main radiation events correspond to the key changes in climate systems in the Pleistocene, namely the MPT, the lengthening of glacial periods from 41 to 100 Kyr, and the initiation of intense glaciation periods (Figs 2, 3, 6). Additionally, there are several branching events that correlate with transitions from an interglacial to glacial period or vice versa. Second, in relation to climatic transitions, altitudinal heterogeneity played a buffer role during climate cycles, allowing populations to cope with severe environmental changes. Thus, the south-western corner of Anatolia, which is more heterogeneous in its topography, altitude, and vegetation than other parts of the region, became a biodiversity hotspot. The present data also demonstrate that effects of the climate cycles of the Pleistocene on populations differ according to the altitudinal and latitudinal locations of the populations in Anatolia. All of these factors together have caused shifts in the habitat preferences of populations/taxa. Third, the terrestrial connection through Dardanelles was an active distribution corridor allowing faunal exchange between Anatolia and the Balkans during the Pleistocene. There are also local barriers, such as the Menderes River, which caused speciation. Fourth, habitat preferences, such as altitudinal range, may easily shift as a result of environmental changes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Populations, haplotype codes, and number of individuals sharing the respective haplotypes for 16S rDNA sequences of the *Poecilimon luschani* species group. Upper case letters indicate haplotypes not used in time estimation analyses; *short sequences of 310 bp, others are 539 bp.

Table S2. Populations, haplotype codes, and number of individuals sharing the respective haplotypes for cytochrome c oxidase subunit I (COI) sequences of the *Poecilimon luschani* species group. (*shared haplotypes).

Table S3. GenBank accession numbers for cytochrome c oxidase subunit I (COI) and 16S rDNA haplotypes, and their concatenation scheme for the data set used in the time estimation analyses of the *Poecilimon luschani* species group. The haplotype codes in upper case letters indicate the haplotypes used in the demographic analyses but not in time estimation. Haplotype accession numbers in bold indicate sequences obtained in this study; others were downloaded from GenBank and published in either Kaya *et al*. (2012) or Boztepe *et al*. (2013).