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„Phylogeography of the plant species *Phlomis olivieri* (Lamiaceae) in the high mountains of Iran“

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Abstract

In this study I focus on phylogeography of *Phlomis olivieri* (Lamiaceae), an endemic plant species distributed in mid elevations of high mountains of the Iranian Plateau. Phylogeography of this area, in general, is poorly known compared to other mountain ranges like the European Alps. Glaciation in this region was not as strong as in Europe, nevertheless its biota was affected by Pleistocene climatic fluctuations. A total of 14 populations with 53 individuals of *Phlomis olivieri* were successfully sequenced (Rad-Seq). Population structure of the species was analysed, and environmental niche modelling was used to analyse habitat suitability of the species in the past and present. Here we identify three well distinguished population genetic groups of the species that are congruent with three areas of endemism corresponding to three main mountain ranges. ENM shows that total habitat suitability of the species during LGM was higher than today but just concentrated to NW Iran and W Zagros. Based on these results we conclude that the major mountain ranges of the region have been biodiversity refugial areas in the LGM.

In dieser Studie konzentriere ich mich auf die Phylogeographie von *Phlomis olivieri* (Lamiaceae) einer endemischen Pflanzenart, die in mittleren Erhebungen des Hochgebirges des iranischen Plateaus verbreitet ist. Die Phylogeographie dieses Gebiets ist im Allgemeinen im Vergleich zu anderen Gebirgszügen wie den europäischen Alpen kaum bekannt. Die Vereisung in dieser Region war nicht so stark wie in Europa, dennoch wurde ihre Biota durch pleistozäne Klimaschwankungen beeinflusst. Insgesamt 14 Populationen mit 53 Individuen von *Phlomis olivieri* wurden erfolgreich sequenziert (Rad-Seq). Die Populationsstruktur der Art wurde analysiert, und die Umweltnischenmodellierung wurde verwendet, um die Habitateignung der Art in Vergangenheit und Gegenwart zu analysieren. Hier identifizieren wir drei wohlunterscheidbare populationsgenetische Gruppen der Arten, die mit drei Endemismusgebieten übereinstimmen, die drei Hauptgebirgen entsprechen. ENM zeigt, dass die Gesamtlebensraumeignung der Art während der LGM höher war als heute, sich aber nur auf den NW-Iran und W-Zagros konzentrierte. Basierend auf diesen Ergebnissen schlussfolgern wir, d dass die großen Bergketten der Region Rückzugsgebiete für die Biodiversität im LGM waren.

Keywords: endemism, environmental heterogeneity, Iranian Plateau, Last glacial maximum, *Phlomis olivieri*, phylogeography, refugium, topographic complexity.

Introduction

Phylogeography is defined by Avise et al. (1987) as a research field that examines the spatial genetic structure of species, and it is concerned with documenting the spatial distribution of genetic lineages. It also focuses on processes and principles governing the geographic distributions of genealogical lineages within and among closely related species. It tries to explain the extent and mechanisms by which historical processes in population demographics have shaped current geographic distributions of organismal traits (Avise et al. 2000). Large number of phylogeographic studies and data across a variety of systems and taxa indicate multiple responses to past climate change and other geological events. Particularly, late Quaternary history of many species was defined by perseverance in relatively small and isolated refugia (Carnaval and Moritz 2009). Phylogeography combined with other concepts and methods across disciplines like niche modelling, can answer questions about distribution of species during different time periods. It can locate potential refugia based on genetic structure of the species, and help us to understand responses to past glaciations and impacts of Pleistocene climate oscillations. (Marske et al. 2016, Pahad et al. 2019).

RAD sequencing uses next generation sequencing to generate sequence data next to a high number of restriction sites, which can occur in all areas of the genome and individuals within or between closely related species (Andrews et al. 2016). These techniques dramatically changed studies in ecological, evolutionary and conservation genomics by making the access to thousands of polymorphic genetic markers simple and cost-effective (Davey et al. 2011, Luikart et al. 2003). RAD sequencing targets a subset of a genome, giving advantages over whole genome sequencing with a bigger depth of coverage per locus and a higher number of samples. Because it does not need prior genomic information for the studied taxa, it has become most used genomic approach for high-throughput single nucleotide polymorphism (SNP) discovery and genotyping in ecological and evolutionary studies of non-model organisms (Andrews et al. 2016).

Iran with its high mountain ranges is located in South-West Asia. Three phytogeographical regions (Zohary et al. 1973) and two global biodiversity hotspots (Mittermeier et al. 2005) reaching to each other in this region. The glaciation in the region was not as strong as Europe, but its biota was severely influenced by Pleistocene climatic fluctuations (Djamali et al. 2008). Despite its rich flora and vegetation (Noroozi et al. 2019), our knowledge about the phylogeography of the region is very poor and limited to some studies on the animal species (Rajaei Sh et al. 2013, Aghbolagi et al. 2019, Amiri et al. 2021, Ghane-Ameleh et al. 2021).

In this study I will focus on *Phlomis olivieri* (Lamiaceae), an endemic plant species distributed in mid elevations of high mountains of the Iranian Plateau. This species is continuously distributed from North-Western (NW) Iran to Zagros and Alborz mountains, but probably such a continuous distribution was not possible during the Last Glacial Maximum (LGM) and it was more likely restricted to the unconnected lowland valleys. With postglacial global warming, it probably moved gradually up and spread all over the montane zone of these mountain ranges. Based on this assumption we would expect different population groups of *P.olivieri* to correspond with three main mountain ranges where this species is distributed. We hypothesise that genetic patterns and differentiated groups will correlate with different mountain ranges because of isolation, topographic complexity, and geomorphological barriers. Based on this hypothesis we used statistical phylogeography based on Rad-Seq data and widely used ecological niche modelling (ENM) to answer following questions: How was the distribution of this species during cold periods? Can we see any potential Pleistocene refugia based on the genetic structure of the populations? As the areas of endemism are results of the past ecological and evolutionary phenomena, we expect to see the congruent patterns in the population genetic structure and floristic patterns. Therefore, are population genetic structures of this species concurrent with areas of endemism and floristic concurrent distribution patterns?

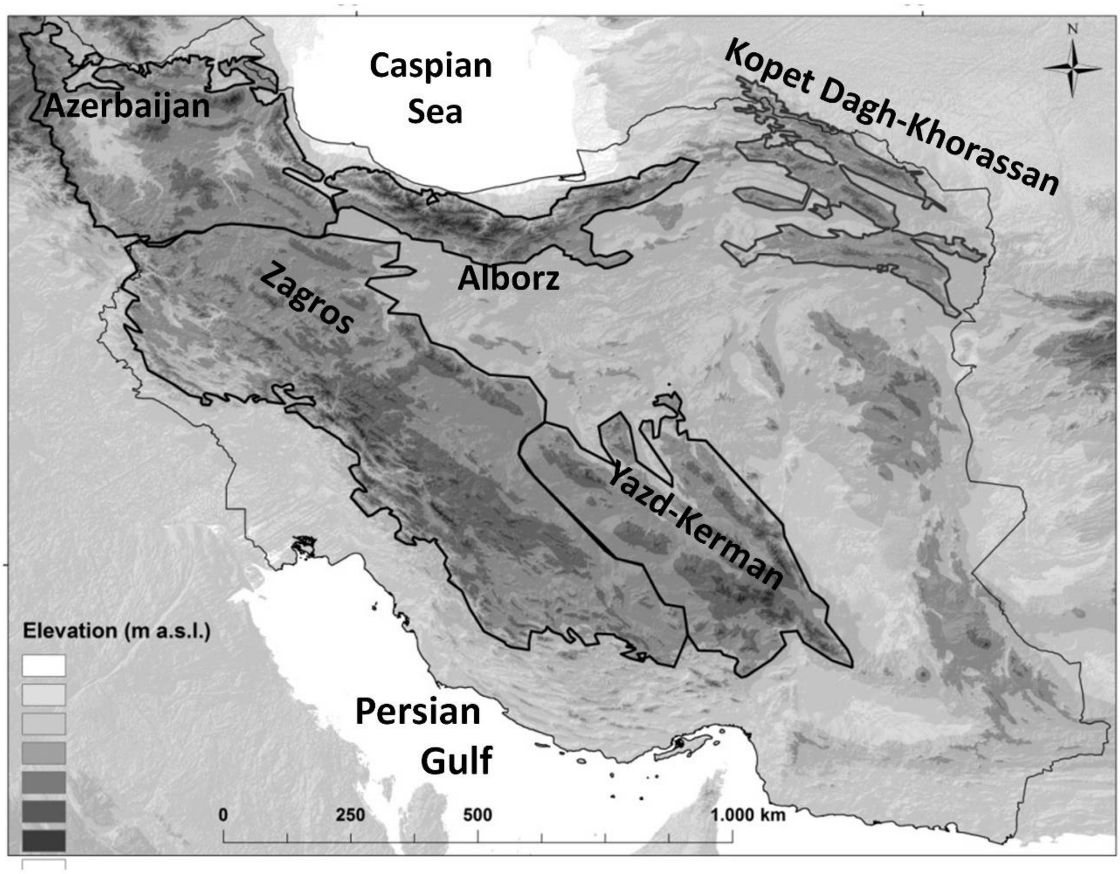

Figure 1. Iranian Plateau with its surrounding mountain ranges and associated areas of endemism (see Noroozi et al. 2019)

Methods

Study area

About half of total surface area of Iran is covered by mountains (Fig. 1). The major mountain ranges are Alborz in the north, Kopet Dagh-Khorassan in the east and north-east, Makran and Baluchestan mountains in the south-east, Yazd-Kerman massif in the south, Zagros in the west and south-west and Azerbaijan Plateau in the north-west. Mountain ranges of the Iranian plateau harbour a very high plant diversity and high endemic species richness (Noroozi et al. 2019). The identified areas of endemism of the region are strongly associated with the mountain ranges (Noroozi et al. 2018, 2019). The Iranian plateau covers around 50% of the Irano-Anatolian biodiversity hotspot. The climate of the region in the Irano-Anatolian part is dry with a Mediterranean precipitation regime (Djamali et al. 2012).

Sampling

A total of 14 populations of *Phlomis olivieri* were sampled across the entire geographic distribution in the Iranian Plateau. Seven population from Zagros, four population from the Azerbaijan Plateau and three populations from Alborz were collected (Table 1). The elevations of sampled populations ranged from 1270 to 3400 m a.s.l. Fresh leaves of four individuals from each population were collected and stored in paper bags and put in silica gel. The leaves were stored until DNA extraction.

Code	Location	Date	Altitude (m)	N	E	Nr. of individuals
Phl332	Kashan, Abyaneh, Mountains in S Abyaneh	04.05.2019	2505	33,5683	51,5978	3
Phl349	Marand to Jolfa	21.05.2019	1270	38,7683	45,5989	4
Phl358	Lasem valley	23.05.2019	2400	35,8303	52,1250	4
Phl363	10 km to Neur Lake	28.05.2019	2200	38,0200	48,5208	4
Phl377	Zanjan to Tarom, Kilvan pass	03.06.2019	2400	36,6919	48,7366	3
Phl381	Kelardasht	14.06.2019	1500	36,5108	51,2621	4
Phl420	N Semnan	12.06.2019	1754	35,6200	53,1250	4
Phl426	Bel Mt., Eqlid	16.07.2019	3080	30,7920	52,7660	3
Phl435	Dena Mts.	20.07.2019	3400	30,8910	51,5060	4
Phl442	Kalar Mt, from Choghachor lake	22.07.2019	2430	31,8840	50,9110	4
Phl449	Lorestan, Ghalikuh Mt. near Oshtorankuh	24.07.2019	3200	33,1240	49,4260	4
Phl478	Lorestan, Khorramabad	2019	1748	33,634	48,02	4
Phl479	Hamedan, Ghanjnameh	2019	2349	34,7222	48,4225	4
Phl481	Belgeis Mts.	2019	1998	36,6642	47,3003	4

Table 1. Information on 14 populations of *Phlomis olivieri*, population code, location, date of sampling, altitude (m), coordinates, number of individuals per population

Extraction

DNA extraction was performed with Invisorb Spin Plant Mini Kit (Invitec Molecular) plus a chloroform step, samples have been purified using NucleoSpin gDNA Clean up kit (Macherey-Nagel) following the manufacturer's instructions. DNA concentrations were measured using Qubit Fluorometer at the Vienna Biocenter Core Facilities, Vienna, Austria.

Library preparation

The RAD libraries were prepared following a protocol adapted from Paun et al. (2016) using Pst1 as a restriction enzyme, for a total number of 56 individuals. The libraries were sequenced using the Illumina HiSeq2000 platform as 100bp single-end reads at the Vienna Biocenter Core Facilities (https://ngs.vbcf.ac.at).

Filtering SNPs from RADseq Data

Demultiplexing of the RAD- library into individual samples according to the respective barcode combinations was performed using the software *BamIndexDecoder v. 1* as part of the program *Ilumina2bam v. 0.03* (https://gq1.github.io/illumina2bam/) for a total of 53 individuals (three individuals from three different populations were discarded because of high missingness).

Assembly of RAD dataset was done using *STACKS 2 v. 2.45.* (Rochette and Catchen et al. 2019). In order to determine the optimal parameters for the assembly, a subsample of the full dataset with one individual per population was used. Assemblies were run under different combinations of parameter settings (m3n1M1, m3n2M2, m3n3M3, m3n4M4, m3n5M5, m3n6M6) to choose the one that returned the most loci present in at least 80% of the individuals. The value of the minimum number of identical reads required for a stack to be formed (setting "m") was set to "m=3", the number of mismatches allowed between stacks within individuals (M) and between individuals (n) was also set to 3 respectively. The final combination of settings (m3n3M3) was chosen based on that the number of loci didn't increase by more than one percent. Full assembly was done using *denovo_map.pl* wrapper, as part of *STACKS 2*, for the best parameters and with all individuals. The SNP selection using *populations*, also part of *STACKS 2*, was done by first filtering out loci with maximally ten SNPs to remove potential paralogs. This was done by creating a file with the ID of loci with maximally ten SNPs, which was then included as whitelist in *populations*. Furthermore the "-R" argument was set to 0.5 which requires SNPs to be present in 50% of the individuals, to remove SNPs that are heterozygotic in more than 65% of the individuals "--max_obs_het 0.65" argument was introduced, to instruct *populations* to remove SNPs that are only variable in one individual (singletons) an "--min-mac 3" argument was set. "--write_random_snp" argument selects one random SNPs from each locus, to avoid linked SNPs. All these arguments were used to produce a VCF file with SNPs ready for analysis. After filtering of the SNPs, a total number of the individuals was reduced to 53 due to the high missingness in some individuals that didn't pass the 30% threshold.

Population structure

The input files were converted with *PGDspider2* from vcf to nexus file format. Neighbour-net tree was calculated with *Splitstree4* program (Huson and Bryant et al. 2006), with distances based on the Hasegawa-Kishino-Yano (HKY) substitution model. For principal components analysis (PCA) "Adegenet" in *R studio* was used (Jombart et al. 2008). Converting the VCF file to a structure file (.stru) was performed with *PGDspider2* program. To estimate the admixture ratio and the number of ancestry groups (number of K), "LEA" in *R studio* was used (Frichot et al. 2015). It uses an underlying algorithm that utilizes sparse non-negative matrix factorization (sNMF) to find the best model (best K) by reducing Least Squares (Lee and Seung et al. 1999). Admixture coefficients for K=1 to K=10 were estimated, maximum number of iterations was set to 2000 and the script run for 100 repetitions per K to find the best run (lowest cross entropy). Plotting the results was performed in *R studio.*

Environmental niche modelling (ENM)

ENM was done using *BIOMOD2* (Thuiler et al. 2009), ensemble platform for species distribution modelling. The ensemble was projected onto climate layers from different time periods (Last glacial maximum, present), to reconstruct the climatic niche of *P. olivieri*. Geographic coordinates of P. *olivieri* from the sampled populations and from previous studies (Noroozi et al. 2019), were used. Bioclimatic variables (2.5 arcmin resolution) were downloaded from "Worldclim1.4" for the past (http://www.worldclim.com/paleo-climate1) and "Worldclim2.1" for the present (https://www.worldclim.org/data/worldclim21.html). The coordinates were subsampled to one coordinate per layer cell with custom R script. Ensemble modelling was run with pseudo absence replicates and evaluation (model) replicates both set to ten. Which meant that it run nine models for ten times ten runs. Total of 900 model runs were then combined into an ensemble and projected. Highly correlated variables were removed.

Results

A total of 14 populations with 53 individuals of *Phlomis olivieri* were successfully sequenced and analysed with 12,92x average depth of coverage for processed samples. The Neighbour-net and the PCA analysis suggest that the most fundamental structure in our dataset is a split into three groups (Fig. 2,3): (I) Northern group comprising populations from north-western Iran (yellow circle; Phl349, Phl363, Phl481, Phl377; Fig. 2); (II) Alborz group (blue circle; Phl358, Phl420); (III) Southern group including populations from Zagros mountains (red circle; Phl478, Phl449, Phl332, Phl442, Phl426, Phl435; Fig.2). Two admixed populations were isolated and separated from the others located in Kelardasht and Alvand regions (Phl381 and Phl479, respectively) (Fig. 2). The Northern and Southern groups were separated along the first axes of the PCA. PC1 explained 10,64 % of the overall variation, while PC2 and PC3 explained 5,85 % and 4,23 %, respectively, with PC2 further splitting the Alborz group from the rest of the northern populations into a separate group, resulting in three well separated groups. (Fig. 3). The sNMF analysis shows that the cross-entropy values for K=2, K=3, and K=4 were relatively equal, but K=3 shows a great deal of genetic admixture located in the contact zone between these three groups.

The ENM for the present predicted suitable areas for *P. olivieri* is mostly matching the current distribution range of the species. The AUC value for the current potential distribution is high (0.954). Current projections show that the suitable habitats for the species are NW Iran, Alborz and Zagros mountains. According to the LGM projections, habitat suitability was very high in NW Iran and NW Zagros and intermediate in western Alborz and poor in central and eastern Alborz and south-eastern Zagros. Total suitability, estimated as the sum of habitat suitabilities, during the LGM was higher than today (ratio of sums of present and LGM habitat suitabilities of 0.68) but it was concentrated in the NW and W Iran.

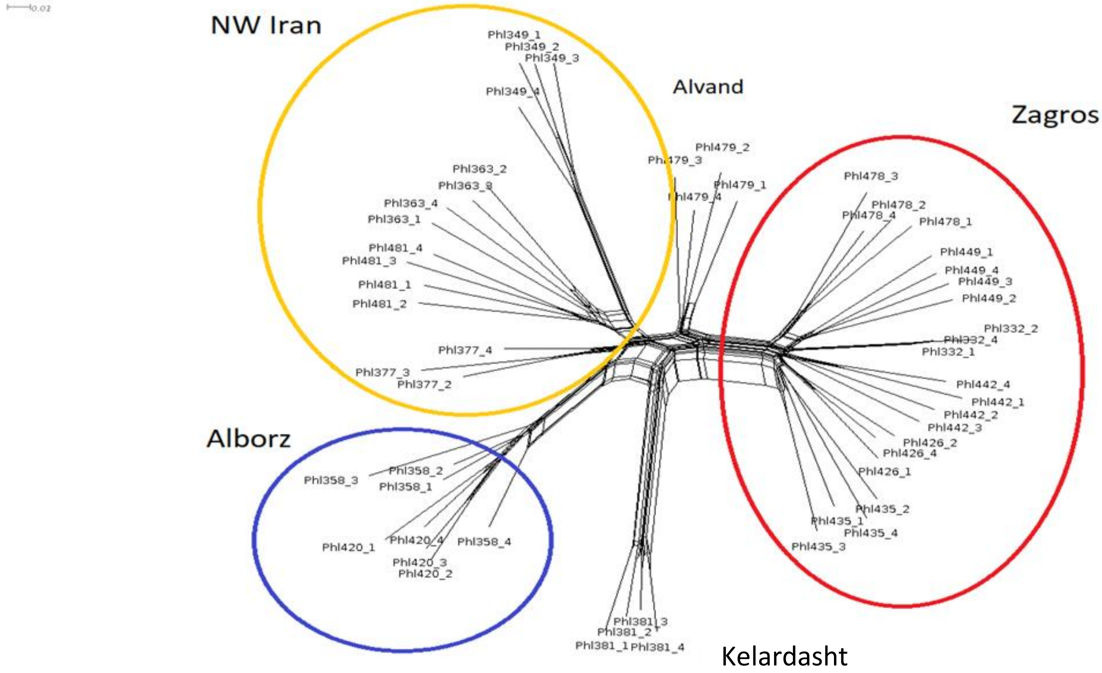

Figure 2. Neighbour-net of 14 populations with 53 individuals, based on SNP data.

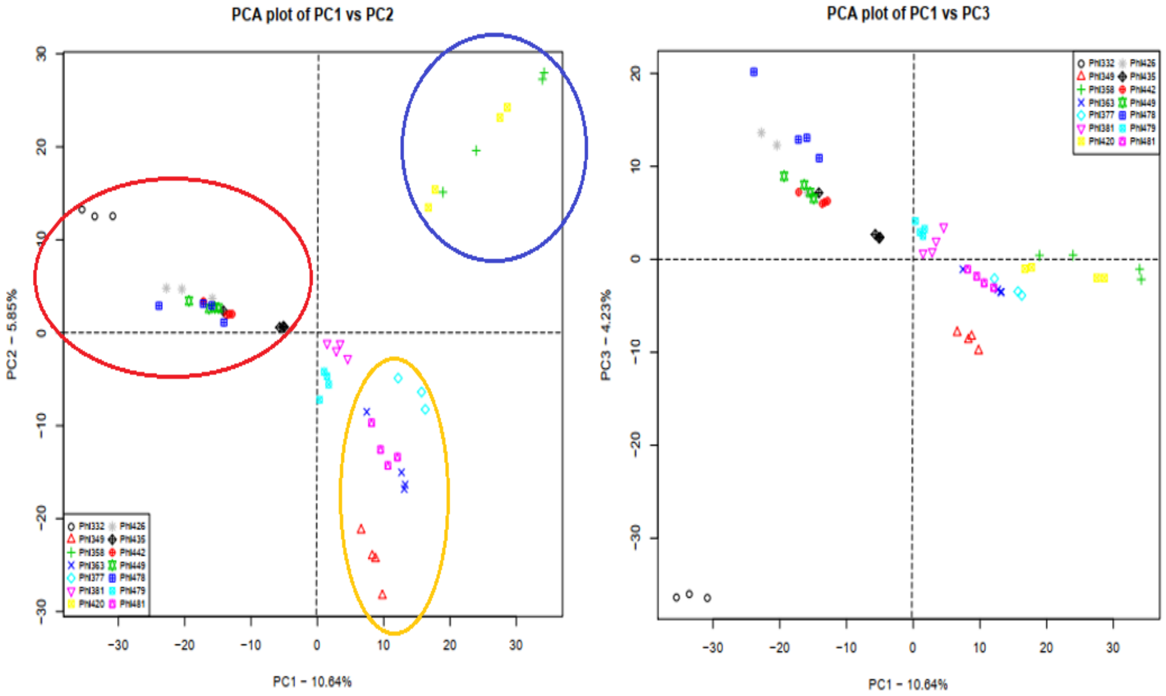

Figure 3. Principle components analysis (PCA) of 53 individuals, ordination axis PC1 vs PC2 and PC1 vs PC3

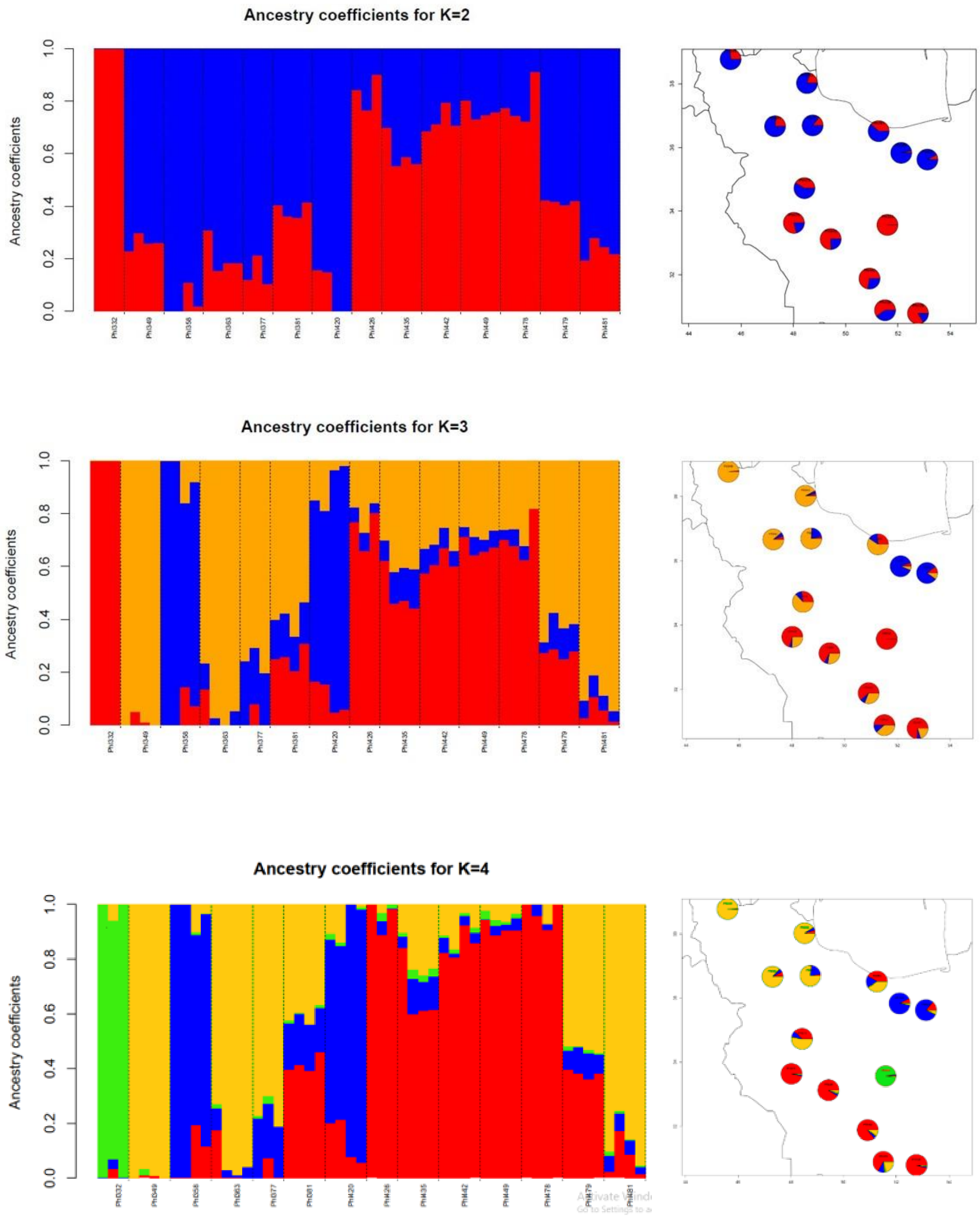

Figure 4. Ancestry coefficients for K=2, K=3 and K=4, admixture ratios of different populations represented as colours in the bar, each individual is one bar in the bar plot (left), and geographical distributions of main groups of *P. olivieri* estimated by sNMF analysis (right)

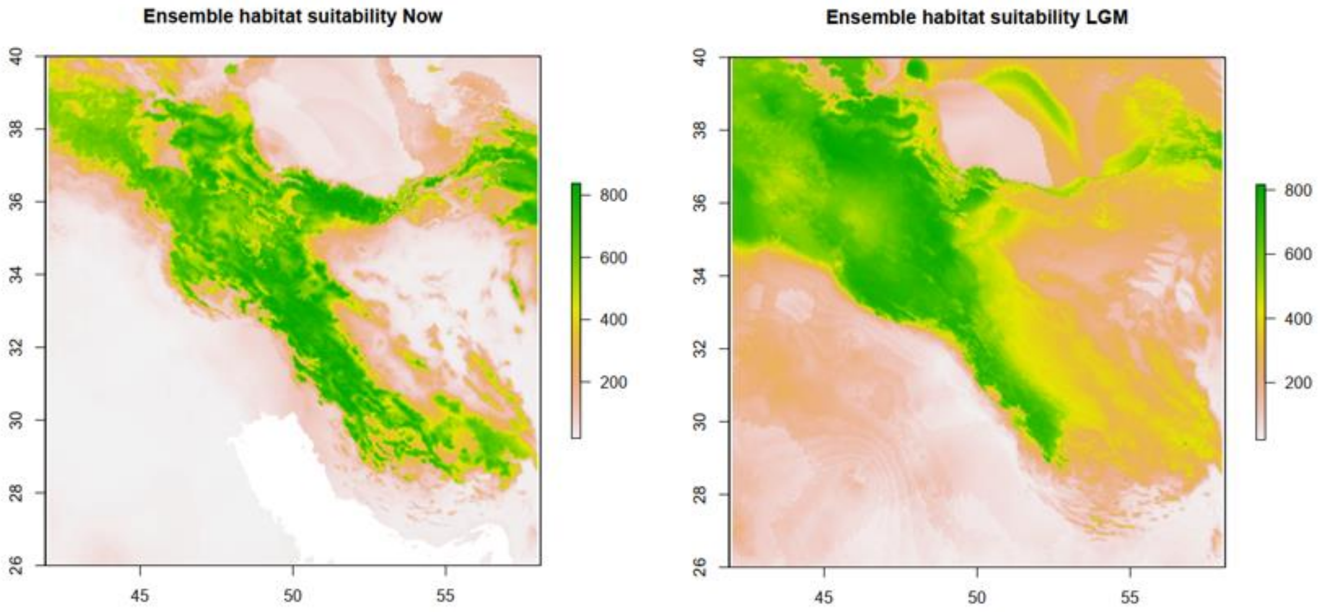

Figure 5. Predicted distributions and habitat suitability for *Phlomis olivieri* (current, LGM)

Discussion

This study represents the population structures and ENM analyses of an endemic mountain species of Iranian high mountains. We identified three well separated genetic groups for *Phlomis olivieri* which are well associated with three areas of endemism corresponding to the major mountain ranges, i.e., Azerbaijan Plateau, Alborz and Zagros (Noroozi et al. 2018, 2019). Well differentiated genetic groups of this species over the species range might indicate long isolation of populations in separate refugia during the LGM. Mountains are important refugial areas, with long-term ecological stability buffering the impacts of the alterations in warm and cold as well as wet and dry periods (Taberlet and Cheddadi 2002). Given the fact that areas of endemism often coincide with refugial areas (Tribsch et al. 2003), we conclude that the congruency of the genetic structure of this species with areas of endemism of the region are result of Pleistocene glacial and interglacial climate fluctuations. High topographic complexity and environmental heterogeneity can increase habitat diversity and create refugia for species during climatic fluctuations (Badgley et al. 2017), and this helps mountains to be refugial areas for the mountain species. This has been already shown and discussed for alpine species of the Alps (Tribsch and Schönswetter 2003, Schönswetter et al. 2005, Schönswetter and Schneeweiss 2019, Surina, Schönswetter and Schneeweiss 2011). Unconnected low mountains and valleys of the Iranian Plateau might have served as a potential glacial refugia for montane species and a starting point for postglacial recolonisation of the Iranian mountain ranges. With interglacial climate warming, the populations probably expanded their range size to mid montane zone and distributed all over the mountain ranges of the region with a continuous distribution pattern.

According to the ENM, it seems like the suitable habitat was quite poor in Alborz and southern Zagros during the LGM and became slightly suitable today in these regions. The ENM shows that the north-western region and Azerbaijan plateau experienced a slight contraction in habitat suitability and a remarkable expansion in east and southwest. The biggest expansion of habitat suitability can be seen around Alborz. The results of ENM are partly in line with the results of population genetic structure and explains why each mountain range has an independent population group especially in Alborz. However, strong connection of Western Alborz and NW

Iran does not support the genetic data. In general, the results of ENM somehow supports this hypothesis that the montane species were in refugia during the LGM and expanded their range size in interglacial periods (Bettin et al. 2007, Birks et al. 2008, Hewitt et al. 2004, Provan and Bennett et al. 2008, Theodoridis et al. 2017). However, the ENM results are only based on climatic data and other factors like topographic complexity, soil structure and general topography, are not included. These factors must have had important impact on the distribution of *P. olivieri*. This might be a reason why ENM results and genetic results are not strongly associated.

In general, regional refugia in NW Iran, Alborz and Zagros mountains might have had an important role in shaping phylogeographical patterns and Holocene recolonization of these mountains. However, we need to study the phylogeography of more species from different elevational zones to have a good and clearer idea about the impacts of the Pleistocene climate oscillation on the range size of the mountain flora of the region. These researches might help us to understand the responses of the mountain flora of the region to ongoing climate change and assist us in the improvement of conservation strategies.

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