

Representation of the Hyrcanian forest (northern Iran) in modern pollen rain revealed by palynological and DNA-metabarcoding data

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ABSTRACT. We studied the modern pollen rain in two different landscapes from Hyrcanian lowland forests up to the slopes of the Alborz Mountains in Gilan province for the first time. Pollen traps were installed for one year and moss samples were collected along two altitudinal transects from 100 to 1800 m and from 100 to 2300 m elevations. The results of pollen counting and environmental DNA barcoding (metabarcoding) of the collected pollen and moss samples were compared from 32 locations. In total, 81 vascular plant species from 36 families were identified by metabarcoding, and 68 taxa belonging to 39 families were identified by pollen counting. The pollen counting results reflect mainly wind-pollinated families, such as Betulaceae and Fagaceae while results from metabarcoding of the *rbcl* and ITS2 loci were more in line with the vegetation around the pollen traps and the moss samples. Furthermore, this study showed that the *rbcl* region is able to identify more taxa than the ITS2 region, while applying both markers provides higher confidence. Also using both metabarcoding and pollen data provides a better local and regional vegetation representation.

KEYWORDS: Hyrcanian forest, modern pollen rain, metabarcoding, ITS2, *rbcl*, palynology

INTRODUCTION

The Hyrcanian forest of Iran occurs between the northern slopes of Alborz and the southern coast of the Caspian Sea (CS) forming the 820 km long narrow belt which occupies 6% of the area in Iran. This region extends from the coastal area up to >2000 m a.s.l. (Sabeti, 1994). However, above 2000 m, the forests are mostly replaced by forest/steppe or steppe vegetation. The area covers approximately 1.9 million ha

and consists of three provinces from west to east, namely Gilan, Mazandaran, and Golestan. This small belt hosts 3234 plant species, including ~500 endemic plants, and accounts for 44% of plant diversity (Akhani et al., 2010).

Along with the forests of northern Anatolia, Hyrcanian forests constitute the most important refugia and the last relicts of broad-leaved deciduous forests that covered the temperate zones of the northern hemisphere before the Quaternary (Leroy and Roiron, 1996; Ramezani et al., 2008).

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During the Pliocene, the Arcto-Tertiary flora covered the entire temperate zone of the northern hemisphere. This flora, however, became extinct in Europe and northern Asia during the Pleistocene. Studies suggest that northern Iran, Caucasus and the southern coast of the Black Sea, might act as a refugium for numerous Arcto-Tertiary species (Browicz, 1989).

As a restricted area between mountains and the sea, the upper forest line and coastal vegetation have been subjected to pronounced changes due to temperature fluctuations, changes in the rainfall regime, and sea level changes of the CS, respectively, in space and time. In addition, due to extensive anthropogenic forces during the last centuries, vegetation has been modified significantly. Occurring in such changeable environmental conditions, investigating the Hyrcanian environment, including vegetation, plant diversity and human impact, is of crucial importance.

Modern pollen rain studies are providing important data with different aims, such as generating environmental and paleoenvironmental parameters or providing insight in the current plant diversity of the area. Furthermore, modern pollen rain data can be used in studies related to paleogeographic reconstruction, allergic diseases (hay fever) and occurrence of plant communities in a region (Gomes et al., 2021).

The first pollen-vegetation investigation of the Hyrcanian forest in the province of Golestan was made by Djamali et al. (2009). Their descriptive approach focused on the forest-steppe transect in Golestan National Park in the eastern Hyrcanian forest. Later, Ramezani et al. (2013) provided a study on the pollen-vegetation relationship along an altitudinal transect with a primary focus on tree distribution and their pollen grain dispersal ability in the central part of the Hyrcanian forest.

Different methods can be applied for pollen identification and classification, including image-based, spectroscopic and DNA-based detection (Landsmeer et al., 2009; Longhi et al., 2009; Rittenour et al., 2012). The identification of pollen by morphological characteristics using a light microscope allows identification to family and genus level, but rarely to species level (e.g. Halbritter et al., 2018). As an alternative to morphological pollen identification, DNA-based technique can increase the taxonomic resolution (Polling et al., 2022). The DNA-based technique relies on the fragments of the

genome (known as barcode). DNA barcodes are detectable in a broad range of taxa (Coissac et al., 2012) on multi-copy plastid (in plants) or mitochondrial genomes, containing similar sequences within taxa with enough variability to identify different species (Campbell et al., 2020). In plants, nuclear ribosomal region ITS and the chloroplast regions of the *rbcL*, *matK* and *trnH-psbA* are used as DNA barcode separately or in combination (Fazekas et al., 2008, 2009; CBOL Plant Working Group 2009; Chen and Vargas, 2010; Hollingsworth et al., 2011).

The application of the DNA barcode technique for pollen identification has to face two main problems. First, the standard barcoding loci are located mostly on the plastid genome, which can be found in several organelles, particularly in the chloroplast (Fujiwara et al., 2010). These plastids are inherited maternally (e.g. Sakamoto et al., 2008), and some studies have suggested the absence of the ptDNA (plastid DNA) in pollen grains (e.g. Willerslev et al., 2003). However, recent studies proved the presence of ptDNA in the pollen (Galimberti et al., 2014; Hawkins et al., 2015; Kraaijeveld et al., 2015; Richardson et al., 2015a), and the application of metabarcoding method on pollen started. The second issue referred to the samples containing a mixture of multiple species, resulting in that Sanger sequencing is not applicable for these samples (Matsuki et al., 2007; Aziz and Sauve, 2008). To overcome this problem, amplicon cloning technique can be used but is requiring intensive work (e.g. Galimberti et al., 2014). Recent developments in high throughput sequencing led to a price reduction. This technique can address the mixed-taxa identification via the barcoding method (Hawkins et al., 2015; Keller et al., 2015; Kraaijeveld et al., 2015; Richardson et al., 2015b; Sickel et al., 2015), known as environmental DNA metabarcoding.

Over the last few years, analysis of the environmental DNA (eDNA) for identifying plant taxa has become more applied in biodiversity research (Taberlet et al., 2012a; Creer et al., 2016; Jarman et al., 2018). The eDNA metabarcoding study, based on High Throughput Sequencing (HTS) compared with Sanger sequencing, is a valuable method, with lower cost, more accurate results, and the ability of parallel multiple taxa identification (Taberlet et al., 2012b). However, many factors, including technical procedure, sample handling and biological materials, can influence metabarcoding

results. Therefore, every step should be taken meticulously (Baksay et al., 2020).

Another application of the metabarcoding technique is representing the presence of taxa. Studies show a correlation between pollen abundance and HTS read frequencies to detect pollen abundance (Hawkins et al., 2015; Richardson et al., 2015; Bell et al., 2017; Porron et al., 2017; Pinöl et al., 2019). However, prior knowledge of pollen productivity is crucial for vegetation – pollen rain relationship using metabarcoding, as well as pollen data.

The main objectives of our study are (1) to study the finger print of modern vegetation as reflected in the pollen rain using DNA metabarcoding and conventional palynology, (2) to compare the possibilities of DNA metabarcoding method vs. pollen counting in biodiversity research, and (3) to investigate the pollen rain of different vegetation zones along altitudinal transects.

STUDY AREA

The study sites are located along two altitudinal transects in the Gilan province of northern Iran. The Asalem-Khalkhal (AS) transect (from 100 to 2300 m a.s.l.) and the Shanderman-Masal (SH) transect (from 100 to 1800 m a.s.l.) are located between latitude 37°N and longitude 48 to 49°E (Fig. 1).

Gilan with a maximum annual precipitation of 1850 mm and mean annual temperature of 16°C is known as the wettest province of Iran. However, precipitation and temperature decrease from west to east and from the coastal plain of the Caspian Sea to the highlands of the Alborz Mountains (Molavi-Arabshahi et al., 2016). Generally, greatest amount of precipitation is received by the area from the coast to ~1500 m a.s.l. with warm and humid summer and moderate winter. Above 1500 m a.s.l. summers are moderate but winters are cold with some snow. Based on the study done by Naqinezhad et al., (2015) on the altitudinal transect in the Rudsar area (Gilan province), the vegetation composition can be classified into three sectors: lowlands (up to 800 m a.s.l.), highlands (up to 1900 m a.s.l.) and ecotone from forest to steppe (above 1900 m a.s.l.).

1. The lowlands up to 800 m a.s.l. include species such as: *Parrotia persica*, *Diospyrus lotus*, *Alnus subcordata* var. *villosa*, *Acer velutinum* and *Ruscus hyrcanus*.

2. The highlands up to 1900 m a.s.l. include species such as: *Acer cappadocicum*, *Carpinus betulus*, *Quercus castaneifolia* subsp. *castaneifolia*, *Tilia platyphyllos* subsp. *caucasica* are dominant at highlands accompanied by herbal species like *Epipactis persica*, *Euphorbia amygdaloides*, *Festuca drymeia*, *Galium odoratum*. The forest ranging from 1000 to 1900 m a.s.l. is occupied mostly by *Fagus orientalis* accompanied

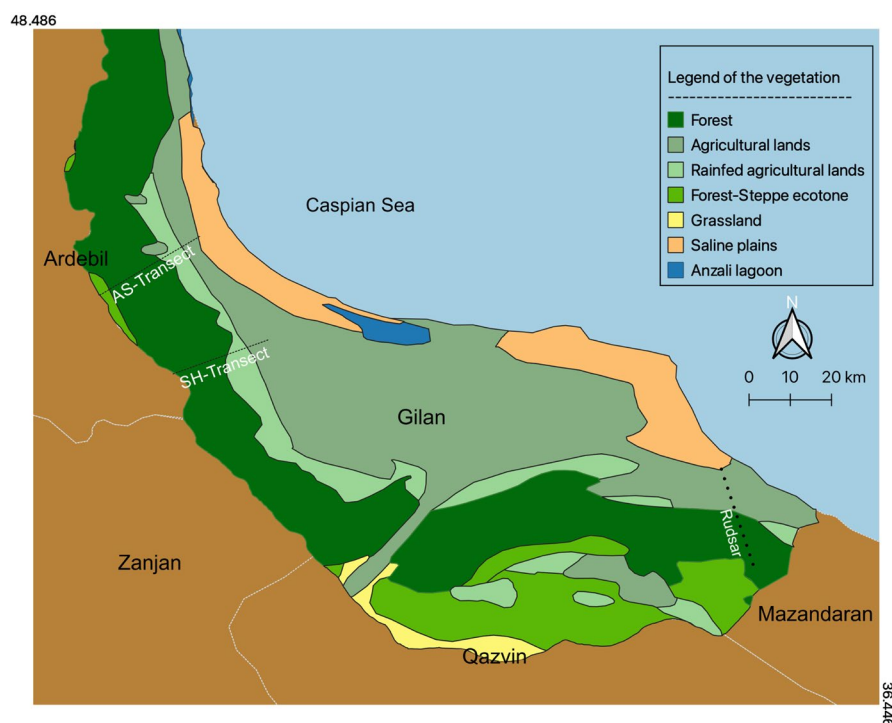


Figure 1. Location of the two transects accompanied by the Rudsar transect (Naqinezhad et al., 2015)

by *Acer velutinum*, *Laurocerasus officinalis* and *Vaccinium arctostaphylos*, *Aruncus vulgaris*, *Brachypodium sylvaticum*, *Cardamine impatiens* var. *pectinata*, *Carex pendula*.

3. The ecotone above 1900 m a.s.l. consists of species such as: *Acer hyrcanum*, *Quercus macranthera* and *Viburnum lantana* which are dominant species mostly accompanied with *Geranium purpureum*, *Lapsana communis*, *Phuopsis stylosa*, *Stachys byzantine* and *Veronica rechingeri*.

MATERIAL AND METHODS

POLLEN RAIN SAMPLING AND MODERN VEGETATION ESTIMATION

In September 2019, 40 Falcon tubes (filled with small amount of synthetic cotton and glycerol and covered by a net to avoid trapping the insects) according to the Behling traps (Jantz et al., 2013) were placed for a year in two different altitudinal transects (from lowlands to highlands) in the Gilan province of Iran. However, only 18 traps could be sampled in September 2020. After failing in finding the Behling traps in the second year, as the replacement of the missing pollen traps, moss samples were collected, wherever it was applicable (a total of 14 moss samples, approximately 25 cm²) (Table 1). Regarding the vegetation along the two transects, a list of the vascular plant species growing within a 5 m radius of each sample was prepared during sampling (for more details see Supplementary File 1¹). Moreover, the percentage of the vegetation cover according to the two categories of arboreal and herbaceous was estimated (Table 2).

A total of 32 collected samples were shipped to the Department of Palynology and Climate Dynamics at Göttingen University, Germany, and stored under dark conditions at 4°C.

Later, samples were sieved with a mesh size of 100 µm, washed with distilled water, and centrifuged at 3500 rpm for 5 min to isolate the pollen grains from other components as far as possible. This method yielded pollen pellets at the conical end of centrifuge tubes. The volume of each sample was increased with distilled water (Behling traps increased to 20 ml and moss traps to 10 ml). Later, 10 ml from Behling traps and 5 ml from moss traps was taken out for the palynological purpose, and the rest remained for DNA extraction.

PALYNOLOGICAL ANALYSIS

Prior to sample processing, one tablet of the exotic *Lycopodium* spores (9.666±212) was added to each Behling trap sample for calculating concentration (grains/cm³). Then the samples were processed based on the pollen analytical methods of Faegri and Iversen

(1989) using chemical treatment (10% HCl, 40% HF and acetolysis). Pollen identification followed the literature (Beug, 2004) and the reference collections of the Department of Palynology and Climate Dynamics.

A minimum of 300 terrestrial pollen grains was counted for each sample. Then, the percentages were calculated on the sum of arboreal (AP) (trees, shrubs, lianas) and non-arboreal pollen (NAP) (grasses and herbs). Based on the pollen sum, the percentage calculation and illustration of pollen were performed using software TILIA and TILIAGRAPH 2.1.1. (Grimm, 1987).

METABARCODING

DNA extraction and amplification

DNA extraction was performed according to the manufacture instructions using NucleoSpin Food Prep Kit from Macherey-Nagel (Düren, Germany). The libraries were prepared using a one-step PCR protocol according to the dual indexing strategy described in Sickel et al. (2015), in short, amplification was performed for two target regions ITS2 and rbcL. The modified sequences of the standard primers for ITS2 (White et al., 1990; Chen and Vargas, 2010) and rbcL (Erickson et al., 2017) were prepared to fit the dual indexing metabarcoding strategy (Sickel et al., 2015). To reduce PCR bias, two separate reactions (25 µl in each) were performed. Also, negative and positive controls with the known species mixture were included in the run. The PCR mixture contained 12.5 µl AccuStart II PCR ToughMix (Quantabio), 1.25 µl 20x EvaGreen (Biotium), 1.25 µl of each ITS2 primer (10µM, biomers), 0.75 µl of each rbcL primers (10 µM, biomers), 2.25 µl PCR grade water and 5 µl DNA (about 20 ng/µl). For the sample-specific labeling, the dual indexing primers with different forward/reverse index combinations were used for each sample. PCR under the following conditions was conducted with the Applied Biosystems 7300 Real-Time PCR System: initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 60 s and elongation at 72°C for 60 s; followed by a final extension step at 72°C for 10 min. The PCR negative control did not yield any sequences. Afterwards, a dissociation curve was used as the control for amplification.

Sequencing

After PCR, the two amplifications product were pooled with equal volumes, 40 µl AMPure beads were added to 50 µl of the mixed PCR product to combine and purify each duplicated sample with magnetic beads (AMPure XP, Beckmann Coulter).

Then samples were washed twice with 80% ethanol, and DNA was eluted in 30 µl of 10 µM Tris (pH 8.5), then the concentration of the mixture was measured using a Qubit fluorometer.

All samples were diluted to 4 nM (except for two with very little amplification which were used without dilution) and pooled in the 4 nM library. Lastly, the library was diluted to 7 pM, denatured, and spiked with 15% of the denature d PhiX Control (Illumina) as described in the 16S Metagenomic Sequencing Library Preparation workflow (Illumina). Finally, sequencing was performed with an Illumina MiSeq sequencer using

¹ Supplementary File 1: Complete list of the identified species during the vegetation survey at the Asalem-Khalkhal transect.

2 × 250 cycles v2 chemistry as described in Sickel et al. (2015). Since different forward/reverse index combinations were used for each sample, the samples were demultiplexed by the MiSeq sequencing software using the dual-index combination.

Data analysis

The subsets of the ITS2 and rbcL markers were split by applying the BLASTn, to estimate the first ten base pairs of each read, which are conserved for each marker and thus are a good discriminator. Then, forward and reverse reads of each marker were quality filtered, dereplicated, denoised and finally merged to amplicon sequence variants (ASVs) using the DADA2 pipeline (Callahan et al., 2016). The quality filtering parameters were set according to the ITS pipeline (Callahan et al., 2016) and pipeline for rbcL diatoms (Keck et al., 2019). The parameters below were set with the same values for both markers:

```
maxN = 0, maxEE = c (2, 2), truncQ = 2,
rm.phix = TRUE
```

As reads belonging to ITS2 show natural length variation between 200–600 base pairs (bp), in order to obtain the maximum sequence diversity, the truncation was not applied to them. Instead, the minimum accepted length of sequences (minLen) was set to 50. Both forward and reverse reads from rbcL were truncated at 240. These numbers were gained from a quality profile plot. ASVs were classified taxonomically against the nr/nt databases of Genbank using the BLASTn (Benson et al., 2009). All ASVs with blast hits below 99% identity score and query coverage were not considered for downstream analysis. If more than one hit has the same quality metrics, the one with the taxon that has

higher frequency among the hits for that sequence was selected and the other hits were classified to the “other possible taxa” group. The possibility of occurrence of these taxa will be checked later using floristic literature and finally the taxa that is more probable from molecular and floristic point of view will be selected.

Key species selection

All assigned taxa (including other possible taxa) were checked in reference books of the flora of Iran (Rechinger, 1963) and in the recent publication Ghorbanalizadeh and Akhane (2021).

STATISTICAL ANALYSIS

All the statistical analyses were done in R, version 4.1.1 (R core team, 2021). First, we calculated the percentage of each taxon per sample at each transect for both metabarcoding and microscopy data. Then illustrated the abundant families (>5% across the entire dataset) as the pie chart for each sample. We also prepared a boxplot in order to compare the numbers of identified taxa between rbcL and ITS2.

RESULTS

ASALEM-KHALKHAL (AS) TRANSECT

Only seven of the 23 Behling traps were found after one year. Wherever it was possible nine moss samples were taken as replacement of the lost Behling traps. Six stations remained without any sample (Table 1).

Table 1. List of the complete pollen traps. Lost samples colored in gray. Note: Prevailing vegetation form is based on the vegetation survey in the field

Asalem-Khalkhal (AS)			Shanderman-Masal (SH)		
Altitude	Prevailing vegetation form	Kind of sample	Altitude	Prevailing vegetation form	Kind of sample
2300	Herbaceous	Trap (dry)			
2200	Herbaceous				
2100	Herbaceous				
2000	Herbaceous	Trap (moist)			
1900	Herbaceous				
1800	Herbaceous	Moss			
1700	Herbaceous	Trap (liquid)	1700	Herbaceous	Moss
1600	Arboreal	Trap (moist)	1600	Arboreal	
1500	Arboreal		1500	Herbaceous	Moss
1400	Arb. & Herb	Trap (moist)	1400	Arboreal	Trap (moist)
1300	Arboreal	Moss	1300	Arboreal	Trap (liquid)
1200	Arboreal	Moss	1200	Herbaceous	Trap (dry)
1100	Arboreal	Moss	1100	Arb. /Her.	Trap (liquid)
1000	Arboreal		1000	Arboreal	Trap (liquid)
900	Arboreal	Moss	900	Arb. /Her.	Trap (liquid)
800	Arboreal		800	Arboreal	Trap (liquid)
700	Arboreal	Moss	700	Arb. /Her.	Trap (liquid)
600	Arb. & Herb	Moss	600	Herbaceous	Moss
500	Arboreal	Moss	500	Arboreal	
400	Arboreal	Moss	400	Arb. /Her.	Trap (liquid)
300	Arboreal	Moss	300	Herbaceous	Moss
200	Herbaceous	Trap (liquid)	200	Arb. /Her.	Trap (liquid)
100	Herbaceous	Trap (moist)	100	Herbaceous	Trap (liquid)

Vegetation survey

Based on the vegetation description along the Asalem-Khalkhal (AS) transect 150 vascular plant species (gymnosperms and angiosperms) have been identified. Between 300 and 1600 m the arboreal plants have higher percentage. Above and below that range the herbaceous plants are dominant (Table 2).

In more detail, in the lowland of the AS transect (up to 800 m a.s.l.) *Alnus subcordata*, *Acer velutinum*, *Carpinus betulus*, *Diospyros lotus* are dominant, together with grasses and herbs like *Brachypodium sylvaticum*, *Parietaria officinalis*, *Rubus hirsutus*.

The main vegetation around the samples in the mountain belt (up to 1800 m a.s.l.) consists of *Acer velutinum*, *Fagus orientalis*, *Alnus subcordata*, *Acer cappadocicum*, *Carpinus betulus* and grass and herbaceous taxa like *Festuca drymeja*, *Brachypodium sylvaticum*, *Galium odoratum*, and *Rubus hirsutus*.

Lastly, in the upper mountain area (above 1800 m a.s.l.) the grass and herbaceous taxa are the most frequent and consist mainly of *Trifolium repens*, *Bromus tectorum*, *Poa trivialis* and *Deschampsia cespitosa* (for more details see Supplementary File 1).

Pollen counting

A total of 54 pollen taxa at different ranks were identified which include 15 families, 1 subfamily (Cichorioideae), 32 genera and 6 species (consisting of *Pterocarya fraxinifolia*, *Juglans regia*, *Polygonum bistorta*, *Polygonum aviculare*, *Plantago lanceolata* and *Plantago major-media*) (Fig. 2).

In the lowland (up to 800 m a.s.l., seven samples) *Alnus*, *Carpinus* and *Pterocarya* with 44.5%, 14% and 9.5%, respectively, compose the most dominant pollen taxa. While, in the highland (up to 1800 m a.s.l., eight samples) *Carpinus* (22%) and *Alnus* (16%) remained the most abundant taxa accompanied by *Fagus* (7%), Poaceae (7%), Amaranthaceae (7%) and *Prunus* (6%). In the upper mountain area (above 1800–2300 m a.s.l., two samples) *Carpinus* (17%), Amaranthaceae (11%), *Quercus* (10%), Poaceae (8%) and Asteraceae (8%) are the main counted pollen.

The most abundant families (>5% of the entire pollen count along the whole transect) are illustrated in Fig. 6 (A). Only seven families represent 85% of the total pollen and consist of Betulaceae 46%, Fagaceae 10%, Poaceae 8%,

Table 2. List of the estimated percent of the vascular plant coverage, within a 5 m radius of each trap

Altitude m (a.s.l.)	Shanderman-Masal (SH)		Asalem-Khalkhal (AS)	
	Arboreal coverage	Herbaceous coverage	Arboreal coverage	Herbaceous coverage
2300	–	–	0	68
2200	–	–	0	80
2100	–	–	0	70
2000	–	–	0	78
1900	–	–	0	73
1800	77	23	45	72
1700	0	95	38	70
1600	75	25	90	15
1500	30	76	84	17
1400	67	33	73	60
1300	70	34	85	18
1200	45	67	80	22
1100	56	48	70	45
1000	75	31	72	41
900	65	60	74	62
800	78	35	70	35
700	70	57	85	20
600	40	75	62	65
500	75	36	73	32
400	50	40	78	37
300	35	67	75	58
200	60	55	20	50
100	40	50	20	55

Rosaceae 5%, Juglandaceae 6%, Asteraceae 5% and Amaranthaceae 5%. The percentages of the pollen count, between elevations 100 and 1600 m, show higher values for the arboreal taxa rather than non-arboreal ones, while between elevations 1700 and 2000 m, they have almost the same frequencies. However, at an elevation of 2300 m, non-arboreal taxa overcome the arboreal ones (Fig. 9).

Metabarcoding

Overall, 99.77% of the reads could be classified to markers, with 67% to rbcL and 33% to ITS2. The separated reads were entered into the DADA2 pipeline to obtain unique sequences (ASVs) and submitted to BLASTn for taxonomy assignment. In total 51,667 reads were assigned to 38 species and four genera (Fig. 3) according to available resources on the flora of Iran and Turkey, among them 59% of the species were identified by rbcL, 35% by ITS2 and 6% by both markers. These species belong to 39 genera and 25 families (see Supplementary Files 2 and 3²).

² Supplementary File 2: Number of reads remained after each step of bioinformatics pipeline in each sample at AS-transects and SH-transects; Supplementary File 3: Comparing the list of the species found within the 5-meter diameter at each elevation point with eDNA results in AS-transect.

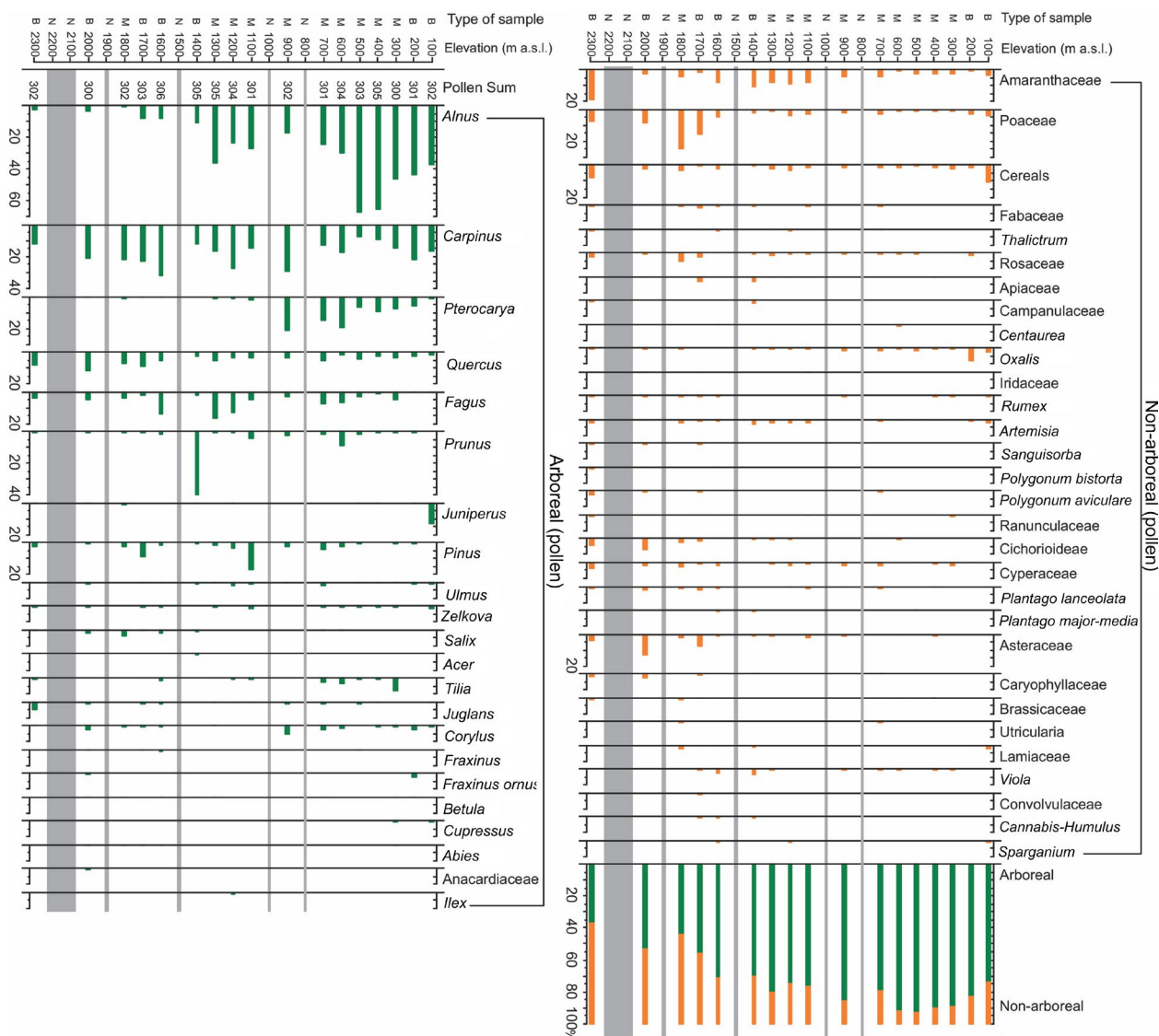


Figure 2. Showing output of the pollen counting method based on the percentage of the most abundant taxa regarding their total pollen sum at each point of the AS-transect. Type of the sample: Behling-trap (B), Mosses (M) and No-sample (N); the lost samples indicated with gray lines

In the lowland (up to 800 m a.s.l., seven samples) *Artemisia annua* (14%), *Carpinus betulus* (12%), *Ulmus minor* (12%), *Alnus* sp. (11%), *Parietaria judaica* (10%) and *Oplismenus undulatifolius* (6%) represent the most abundant reads. At the mountainous area (up to 1800 m a.s.l., eight samples) *Fagus orientalis* (31%), *Ulmus minor* (11%), *Carpinus betulus* (8%), *Trifolium paratense* (8%) and *Taraxacum officinale* (6%) provide the highest reads. However, at the upper mountain areas (1900–2300 m a.s.l., two samples) the dominant reads belong to *Achillea* sp. with 81% and *Silybum marianum* with 6%.

Along the whole transect six families consist of 80% of the total reads and Fig. 6B represents

the contribution of each sample among those six families. These families include Asteraceae (28%), Fagaceae (16%), Betulaceae (15%), Ulmaceae (10%), Poaceae (6%), Urticaceae (5%).

From 100 to 300 m and 1700 to 2300 m a.s.l., the majority of reads belong to non-arboreal taxa, while between 400 to 1600 m, the reads belong mostly to arboreal taxa (Fig. 9).

SHANDERMAN-MASAL (SH) TRANSECT

Of the 17 pollen traps, 11 Behling traps were found after one year. Four moss samples, as the replacement of the lost Behling traps were used, while two stations remained without any sample (Table 1).

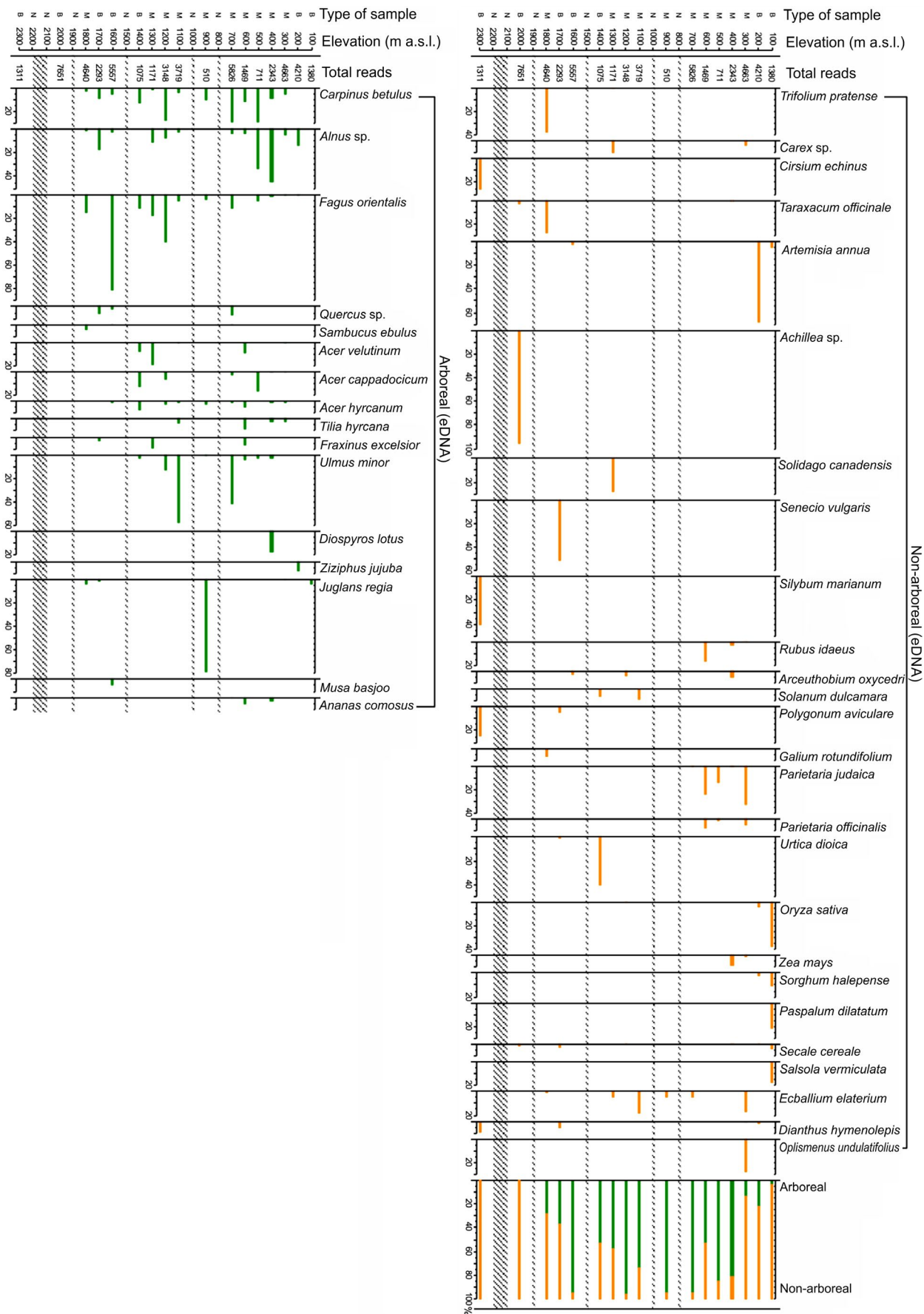


Figure 3. Showing output of the metabarcoding (eDNA) method based on percentage of the most abundant taxa regarding the total number of reads at each point of the AS-transect. Type of sample: Behling-trap (B), Mosses (M) and No-sample (N); the lost samples indicated with gray lines

Vegetation survey

According to the vegetation survey along the Shanderman-Masal (SH) transect, 107 vascular plant species (gymnosperms and angiosperms) have been identified.

In the lowland of the SH transect (up to 800 m a.s.l.) *Carpinus betulus*, *Alnus subcordata*, *Acer velutinum*, *Quercus castaneifolia*, *Oplismenus undulatifolius* have the greatest frequencies.

While, the main vegetation around the samples in the mountain belt (up to 1700 m a.s.l.) consists of *Alnus subcordata*, *Fagus orientalis* and *Carpinus betulus* are the dominant arboreal species. *Clinopodium umbrosum*, *Carex divulsa*, *Tanacetum parthenium*, *Brachypodium sylvaticum* and *Prunella vulgaris* are the most frequent grasses and herbs (for more detail see supplementary).

Pollen count data

A total of 58 taxa at different ranks were identified, which include 15 families, one sub-family (Cichorioideae), 35 genera and seven species (consisting of *Pterocarya fraxinifolia*, *Juglans regia*, *Polygonum bistorta*, *Plantago lanceolata* and *Plantago major-media*, *Ephedra distachya*, *Fraxinus ornus*) (Fig. 4).

In the lowland (up to 800 m a.s.l., seven samples) *Alnus* (33%) *Carpinus* (22%) and *Pterocarya* (9%) provide the most abundant pollen. However, in the highland (above 800 m a.s.l., eight samples) *Alnus*, with 40%, and *Carpinus*, with 29%, are the most dominant pollen.

The most dominant families (only those >5% of the entire pollen count) are shown in Fig. 7A, which represents 83% of the grains, including four families of Betulaceae (64%), Juglandaceae (8%), Fagaceae (7%), Asteraceae (4%). The remaining 32 families (each with less than 5%) provide 17% of the dataset.

Based on the percentage of the pollen counts (Fig. 10), the arboreal and non-arboreal taxa show differences. However, the arboreal pollen was dominant in all samples.

Metabarcoding data

In total 99.85% of the reads were classified to markers, with 60% to rbcL and 40% to ITS2. The taxonomic assignment was the same as in the case of the AS transect. Then, the probability of occurrence was checked for each taxon with the flora of Iran and Turkey. In total, 149,079 reads were assigned to 48 species and

three genera (Fig. 5); 45% of the taxa belong to rbcL, 30% to ITS2 and 25% to both markers, assigning them to 45 genera and 25 families (see Supplementary File 2 and 4³).

In the lowland (up to 800 m a.s.l., seven samples) the most abundant reads belong to *Pisum sativum* (24%), *Carpinus betulus* (12%), *Alnus* sp. (12%) and *Quercus* sp. (12%), while in the highland (above 800 m a.s.l., eight samples) *Carpinus betulus* (27%), *Alnus* sp. (23%), *Acer hyrcanum* (14%) and *Anthemis* sp. (8%) represent most reads.

The proportion of the most abundant families (>5% of the whole transects' reads) is shown in Fig. 7B. Among the 25 families, only six families (including Betulaceae – 33%, Fabaceae – 17%, Fagaceae – 12%, Brassicaceae – 8%, Sapindaceae – 6%, Asteraceae – 4%) account for 80% of the results and the other 19 families provide the remaining 20%.

At 200 m and from 600 to 1300 m elevation, arboreal taxa have greater values than non-arboreal ones, while at 100 and 300 m, the results are vice versa, and non-arboreal taxa have greater values (Fig. 10).

DISCUSSION

COMPARISON OF THE TWO TRANSECTS

We tried to collect the Behling traps for two years, but we failed as they got lost. According to the limitation of time in the project, we decided to collect moss samples, which are known as the natural traps. However, it was not possible to find mosses everywhere. At each point we had only one trap type (Behling or moss), therefore it was not applicable to compare the results of both trap types together in both methods (pollen counting and metabarcoding), and this is postponed to future related studies.

Asalem-Khalkhal (AS) transect was up to 2300 m a.s.l., consisting of different vegetation types, from lowland agricultural areas to Hyrcanian forests and grasslands in the upper mountains. The constructed road changed the natural composition of the area. The Shanderman-Masal (SH) transect, with notably lower human impact, has greater forest areas than AS.

³ Supplementary File 4: Comparing the list of the species found within the 5-meter diameter at each elevation point with eDNA results in SH-transect.

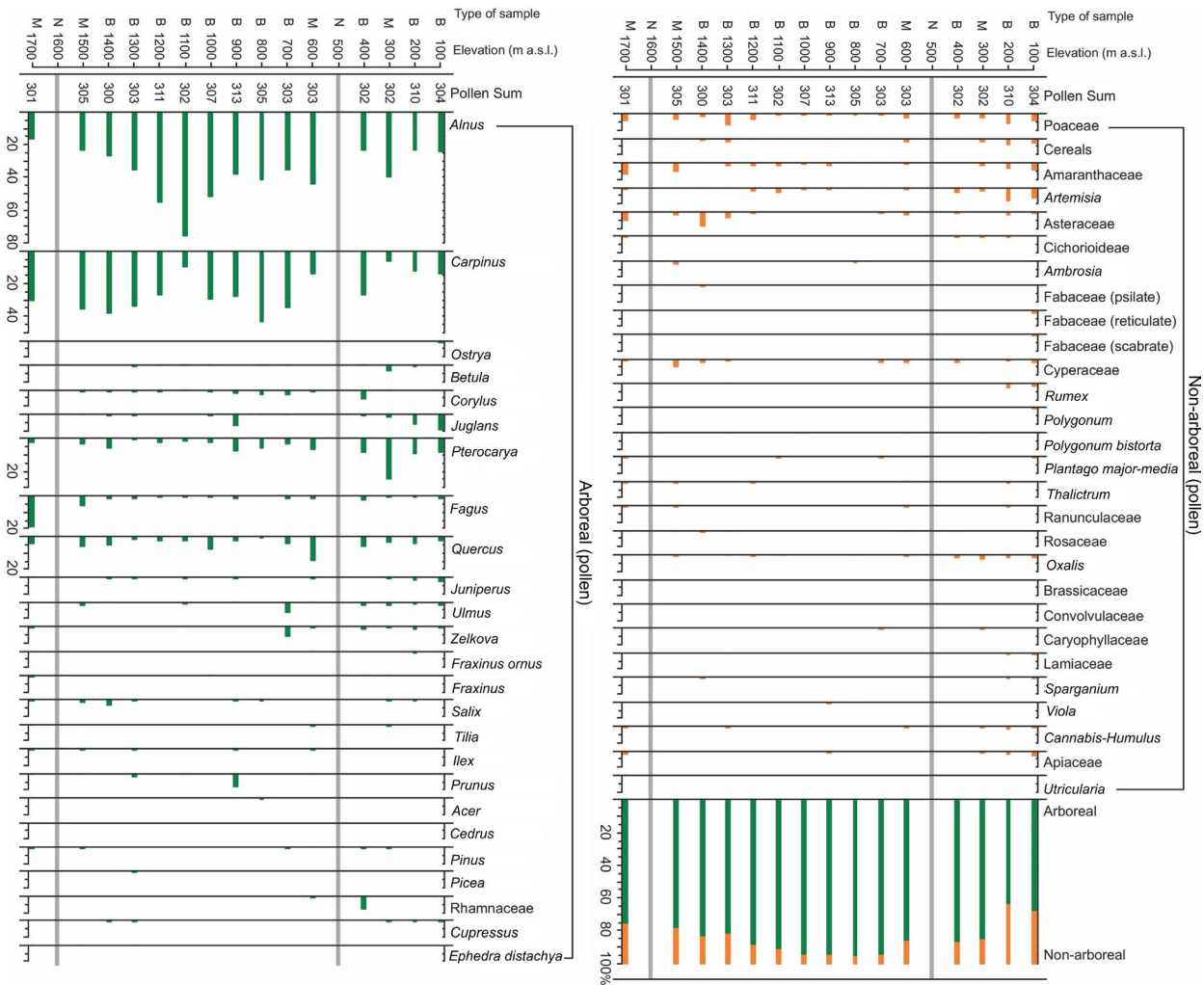


Figure 4. Showing output of the pollen counting method based on the percentage of the most abundant taxa regarding their total pollen sum at each point of the SH-transect. Type of the sample: Behling-trap (B), Mosses (M) and No-sample (N); the lost samples indicated with gray lines

Pollen with different shapes, sizes, weights and pollinating mechanisms have different chances to spread local (not far from the parental plant) or regional (flying more than several kilometers away). Comparing the results of metabarcoding with those of pollen counting, and both results with those of the vegetation survey, could verify that metabarcoding reflects more local vegetation while pollen counting results reflect more regional vegetation. It is possible that the small particles (like leaves) of the surrounding plants drop into the traps and report the presence of that taxa in the dataset, nevertheless each taxon to be detected by metabarcoding needs a minimum quantity DNA. Therefore, if a pollen flew from far away and dropped into the pollen trap it is not possible to detect it by metabarcoding but it still has a chance to be counted in the microscopic observation.

Also, comparing the two transects, indicate that the different zonal mountain vegetation along both transects is well reflected in the metabarcoding results of the pollen rain.

Metabarcoding

At AS transect the Behling traps were mostly lost or dried out and did not have good conditions. However, 44 taxa belonging to 25 families were still recognized. Taxa of six families were more abundant in the transect (Fig. 6B). From 100 to 600 m a.s.l. and above 1700 m a.s.l., herbaceous plants from Poaceae, Asteraceae, and Urticaceae were dominant. Between 700 and 1600 m a.s.l., taxa of Betulaceae, Ulmaceae and Fagaceae were more abundant.

The presence of *Musa basjoo* (Figs 3 and 5) and *Ananas comosus* (see data accessibility link for AS transect) may be the result of the presence of greenhouses in neighboring areas.

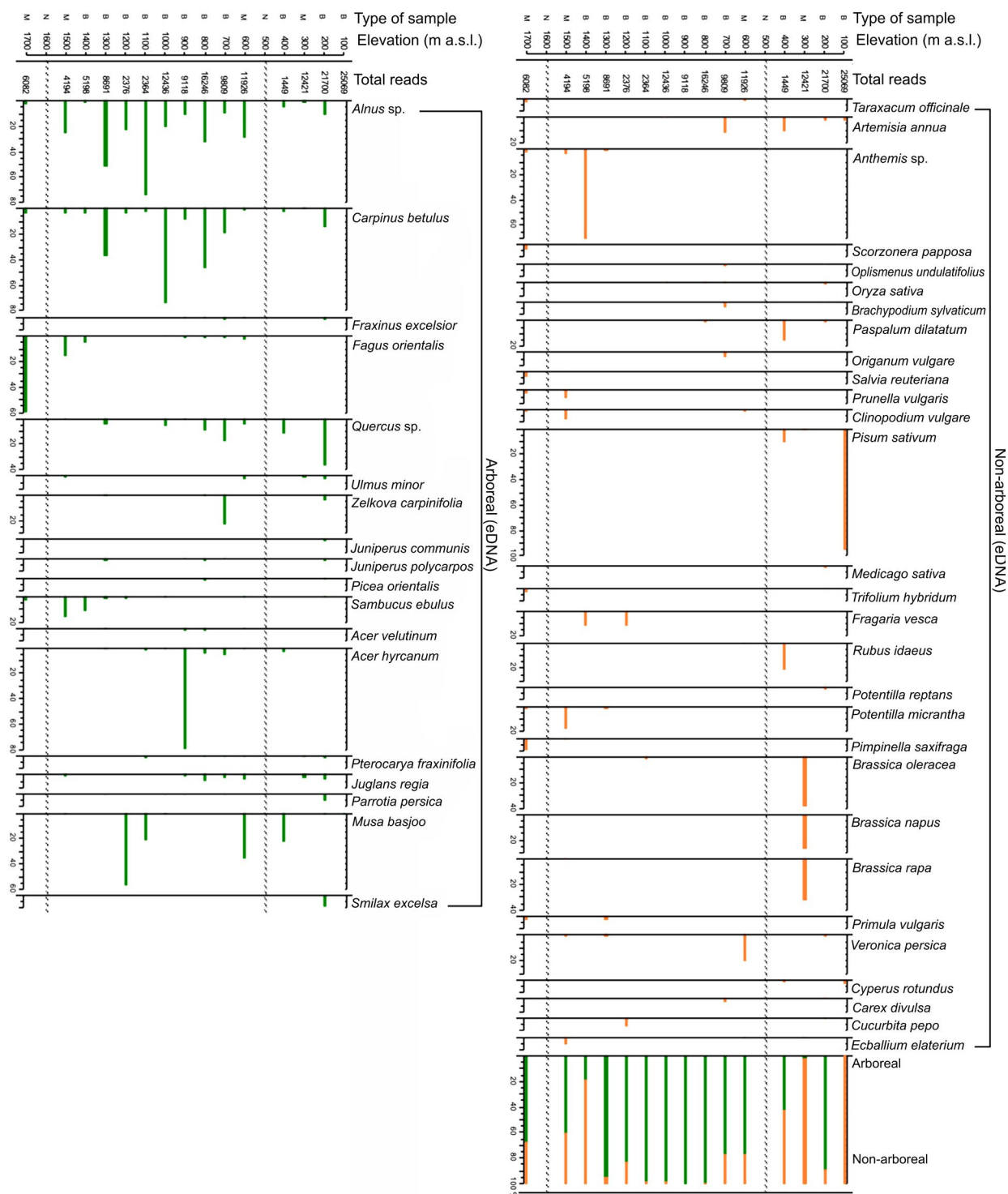


Figure 5. Showing output of the metabarcoding (eDNA) method based on percentage of the most abundant taxa regarding the total number of reads at each point of the SH-transect. Type of sample: Behling-trap (B), Mosses (M) and No-sample (N); the lost samples indicated with gray lines

In the SH transect, 54 taxa belonging to 25 families were identified. Six families are the most abundant ones. Majority of the reads belong to the *rbcL* region (Fig. 8). Up to 400 m a.s.l., taxa from Brassicaceae, Fabaceae and Asteraceae were abundant. Above 400 m, taxa of the Betulaceae, Fagaceae, Sapindaceae and Asteraceae were dominant (Fig. 7B).

Important taxa for the lowland zone (up to 800 m a.s.l.) are *Carpinus betulus*, *Alnus glutinosa*, *Ulmus minor* and *Artemisia annua*, *Parietaria judaica* and *Pisum sativum*. For the mountainous zone (up to 1800 m a.s.l.) the most frequent taxa are *Alnus glutinosa*, *Fagus orientalis*, *Carpinus betulus*, *Acer hyrcanum*.

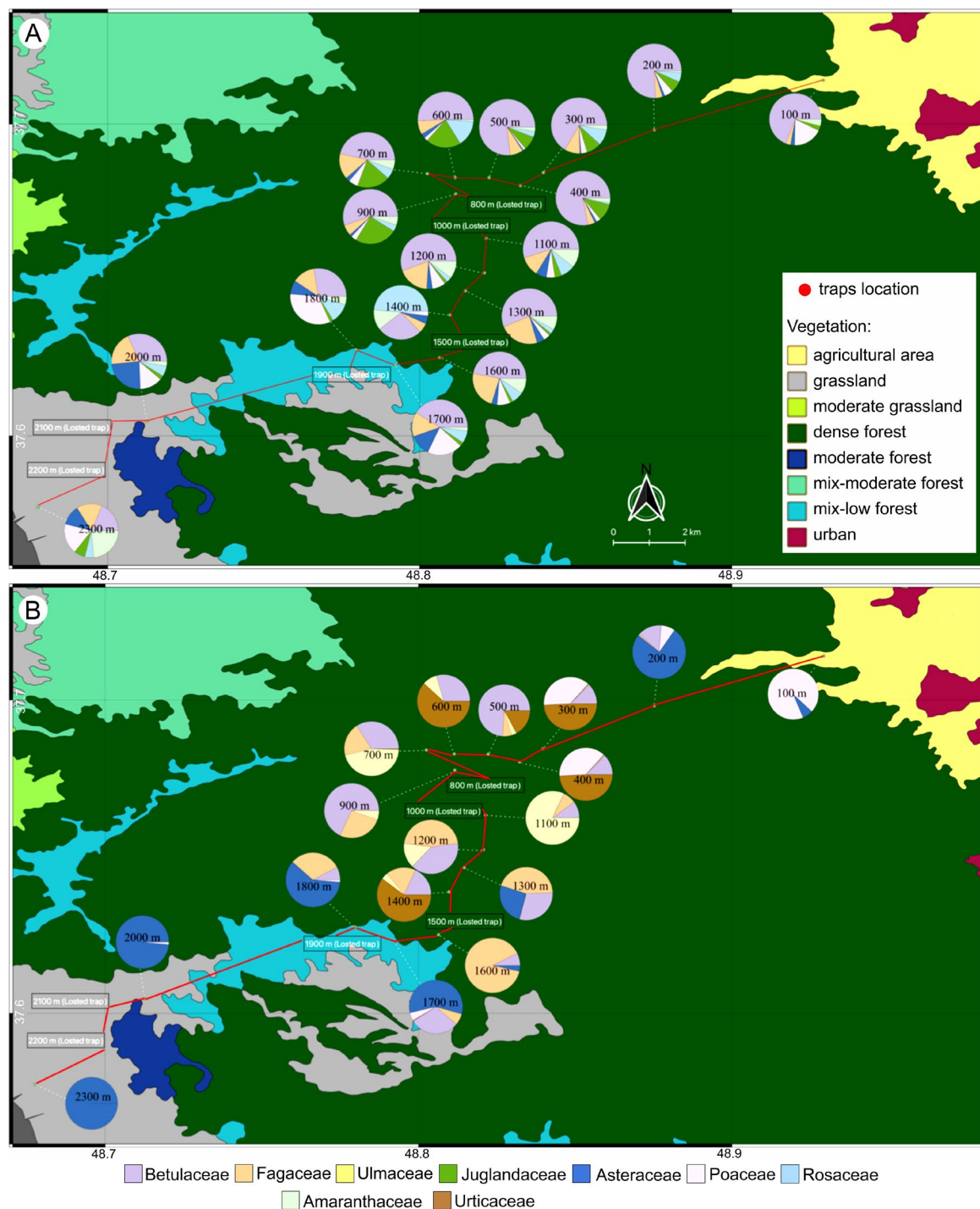


Figure 6. The most abundant families (>5% of presence through the whole AS-transect). **A.** Pollen counting; **B.** metabarcoding reads

The most important upper mountain taxa (up to 2300 m a.s.l.) are only based on the AS transect. These are *Achillea* sp., *Silybum marianum*, *Polygonum aviculare* and *Cirsium echinus*.

Palynology

A total of 54 pollen taxa (at different ranks) were identified in the AS transect. The identified taxa belong to 35 families from which seven families showed higher values along

the transect (Fig. 6A). According to the pollen counting data, from 100 to 1700 m a.s.l., arboreal plants mostly from Betulaceae, Juglandaceae and Fagaceae prevailed, while above 1800 m non-arboreal plants (like Asteraceae, Poaceae and Rosaceae) were dominant (Fig. 9).

Along the SH transect, a total of 58 pollen taxa (at different ranks) were identified, which belong to 36 families. However, four families had more representatives along the transect.

Taxa of Betulaceae were the most abundant ones (Fig. 7A). Based on the percentage of pollen counts (Fig. 10), the arboreal pollen taxa in were dominant all the samples.

HIGHLIGHTS AND CHALLENGES OF THE METHODS

The incomplete plant reference databases of the occurring plants in Iran and the missing pollen traps reduce partly the clarity of the results. Therefore, the interpretation of the results needed to be made with caution. However, the results of the pollen DNA

metabarcoding, besides the pollen counting, are valuable to identify the main plant taxa along the two transects. Some of the advantages and disadvantages of these approaches are discussed below:

1. Although, the number of records from the Iranian plants in GenBank database was relatively low, and made the taxonomic assignment section challenging, the DNA metabarcoding identified, in total, 81 species from 36 families.

2. In metabarcoding, except for a few taxa that were present in most of the samples, the majority of the taxa were rarely detected in more than three samples, also the reference



Figure 7. The most abundant families (>5% of presence through the whole SH-transect). A. Pollen counting; B. metabarcoding reads

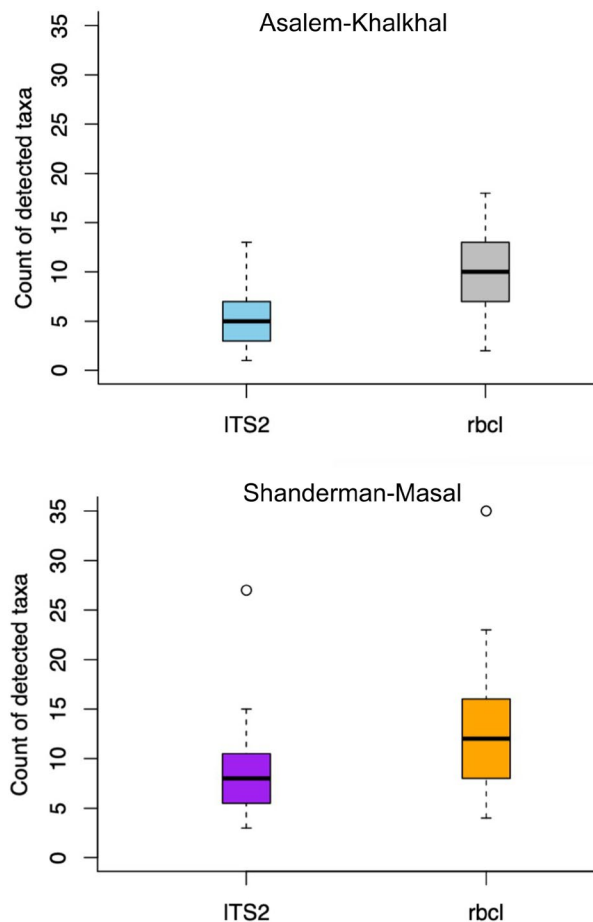


Figure 8. Comparison of the detected species per sample between molecular markers of ITS2 and rbcL at both AS and SH-transects

sequence for some taxa might not be present in the database, therefore it is likely that closely related taxa were assigned as the source. This represents the efficiency of the method for investigating the composition of the vegetation.

3. In this study, two different markers ITS2 and rbcL were used, and taxa identified with the rbcL were slightly higher along both transects (Fig. 8). However, applying both markers provided more evidence of the vegetation cover of the area.

4. The last relicts of the deciduous broad-leaved species *Pterocarya fraxinifolia* and *Zelkova carpinifolia* (Leroy and Roiron, 1996) were present with low frequency in several samples of both transects in the case of both methods.

5. Failure of collecting the samples indicates that one year is too long for traps. We suggest for the future studies to collect the traps collected within a maximum of six months.

6. Regarding the pollen counting method, identification of taxa faced some limitations

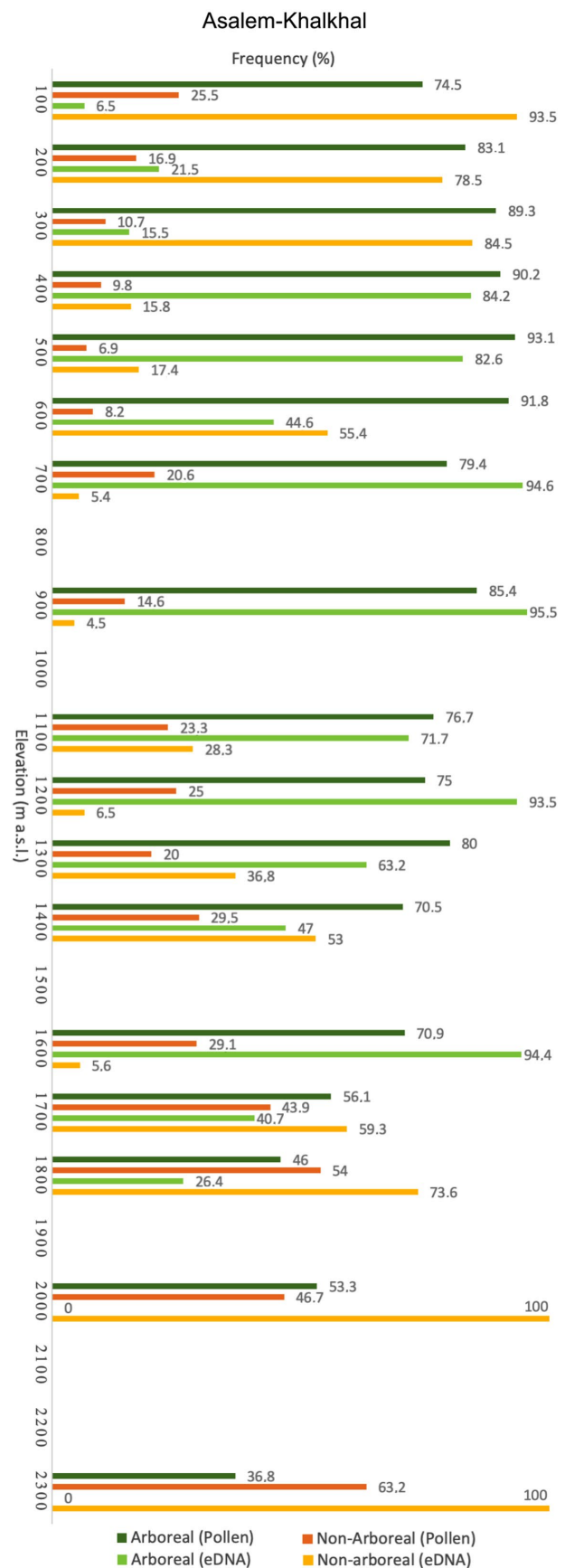


Figure 9. Comparing the prevailed vegetation form (arboreal/non-arboreal), according to the percentage of their presence at each method (eDNA and pollen counting) at different elevation of the AS-transect

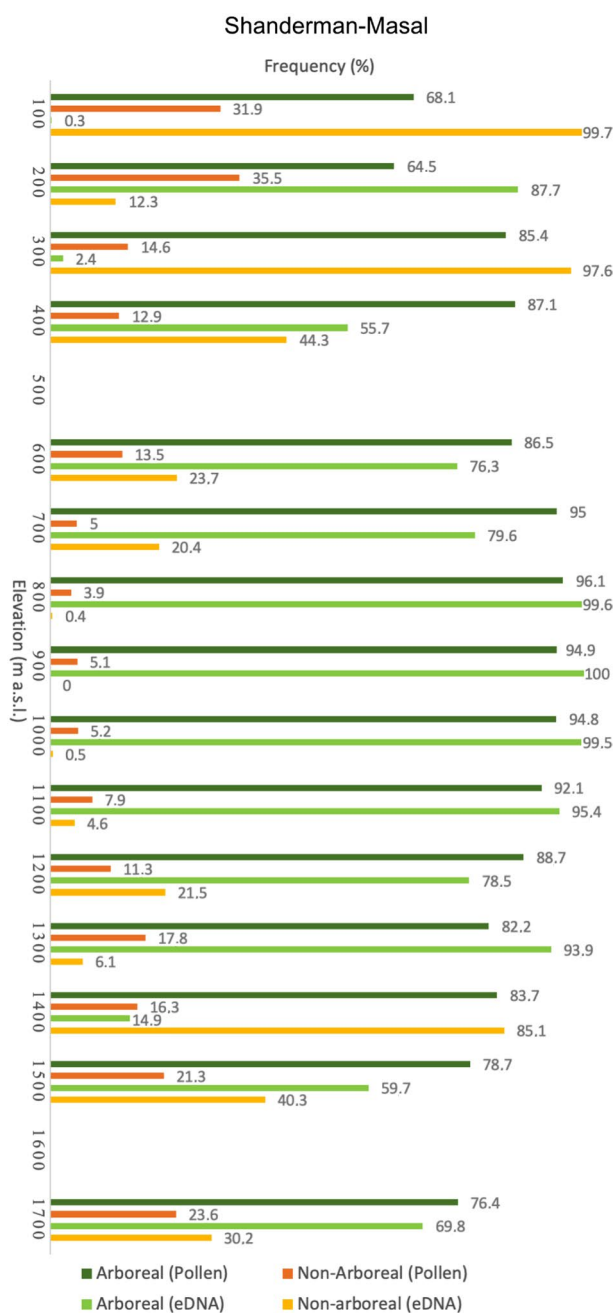


Figure 10. Comparing the prevailed vegetation form (arboreal/non-arboreal), according to the percentage of their presence at each method (eDNA and pollen counting) at different elevation of the AS-transect

like it rarely went down to species level, while metabarcoding, except for some rare cases, identified taxa mainly at species level.

7. The pollen counting approach defined arboreal taxa as the most abundant plants in both transects (Figs 9, 10), which is not supported by DNA results and the vegetation survey.

8. In general, anemophilous taxa like *Carpinus*, *Alnus* and *Quercus* were generally more frequent in pollen counting than the taxa with lower pollen production. Those mentioned genera were present in almost all

the counted samples consistently even in the samples of the non-forested area. Also, counting pollen slides to a high number of pollen grains, such as more than 300 pollen per sample, is time consuming and the time for the pollen analysis was somehow limited (e.g. within this project).

CONCLUSION

This study provided the first metabarcoding insight into the vegetation composition of two altitudinal transects at the western part of the Hyrcanian forest, Iran. DNA metabarcoding of pollen grains can be a robust method which represents vegetation finger print in the pollen rain. Thus, our results can be a first step forward in the nature conservation of the Hyrcanian forest.

However, vegetation reconstruction requires further studies to obtain a better estimation. Also, further studies need to be done to increase and improve the number of species at the dataset's library. Despite the missing pollen trap samples, we were still able to identify 81 different vascular plant species from 36 families, demonstrating the technique's ability to identify species and its application in biodiversity studies.

Lastly, *rbcL* showed more superiority in species identification than *ITS2*. However, applying both markers provides more robust evidence of the vegetation cover of the area.

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DATA ACCESSIBILITY STATEMENT

DNA sequences data are available at: https://drive.google.com/drive/folders/1FYgnAoVzmh9skGaxeNVX_XLaK_IUhwyc?usp=sharing

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