

Research Article



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A new method to quantify atmospheric Poaceae pollen DNA based on the *trnT-F* cpDNA region

Atmosferik Poaceae polen DNA miktarını belirlemek için *trnT-F* cpDNA bölgesini temel alan yeni bir metot

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Abstract

Background: Pollen, mold spores, bacteria and viruses are the main biological substances in the atmosphere causing allergic symptoms and disease. Distinguishing pollen and spores is quite time consuming and requires a trained expert. There is a different approach to identification of these substances such as microscopic analysis. However, DNA based identification of these is becoming popular recently.

Objective: We evaluated the correlation between the quantity of DNA, which was amplified using *trnT-F* cpDNA specific primers in samples obtained from a high volume air sampler (HVAS), and concentration of Poaceae pollen collected with a Burkard trap.

Materials and methods: Here, we present a method for identifying and quantifying airborne Poaceae pollen using a single step polymerase chain reaction (PCR) technique.

Forty daily air samples were collected by HVAS. The method was optimised using two different methods (M1 and M2) and the *trnT-F* cpDNA region was amplified using a Poaceae specific primer pair. The correlation between the quantity of DNA and pollen concentration was tested using R statistical programming language.

Results: Although a significant correlation was obtained between the M1 and M2 methods ($R^2 = 0.655$, $p < 0.01$), the M2 method was more correlated with pollen concentration. The correlation between pollen and DNA content changed due to episodes that were observed during the pollen season. DNA concentrations from the PCR data were significantly correlated with pollen concentrations determined by light microscopy ($R^2 = 0.767$, $p < 0.01$) in episode II using the M2 method and during the entire season ($R^2 = 0.469$, $p < 0.01$) using M2.

Conclusions: The M2 method correctly identified Poaceae pollen in mixed air samples from Zonguldak Province. The non-coding *trnT-F* cpDNA region was used for the first time in aerobiological samples to identify Poaceae pollen. Use of this method that does not require DNA extraction may be a crucial step for real-time pollen monitoring devices to be developed in the future. The correlation strength between pollen and amplified DNA content could be improved using a sampler that has a lower absorption rate, and a more sensitive technique, such as qPCR.

Keywords: PCR; Pollen identification; *trnT-F* region; DNA quantity; Poaceae.

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Öz

Amaç: Atmosferdeki polen, küf sporları, bakteriler ve virüsler alerjik semptomlara ve hastalıklara neden olan

temel biyolojik maddelerdir. Polen ve sporların ayırt edilmesi oldukça zaman almakta ve eğitimli uzman gerektirmektedir. Bu maddelerin teşhisi için mikroskopik analiz gibi farklı yaklaşımlar bulunmaktadır. Bununla beraber, son zamanlarda bunların DNA temelli teşhisi popüler hale gelmiştir. Bu çalışmada Yüksek hacimli hava örnekleyicisinden (HVAS) elde edilen örneklerde *trnT-F* cpDNA'ya özgü primerler kullanılarak amplifiye edilen DNA miktarı ile Burkard tuzağı ile toplanan Poaceae polen konsantrasyonu arasındaki korelasyon değerlendirildi.

Gereç ve Yöntem: Burada, tek aşamalı polimeraz zincir reaksiyonu (PCR) tekniği kullanılarak havadaki Poaceae poleni tanımlamak ve ölçmek için bir yöntem sunulmuştur. HVAS tarafından toplanan kırk günlük örneklerde, iki farklı yöntem optimize edildi ve *trnT-F* cpDNA bölgesi, Poaceae'ye özgü bir primer çifti kullanılarak çoğaltıldı. DNA ve polen konsantrasyonu arasındaki korelasyon, R istatistik programlama dili kullanılarak test edildi.

Bulgular: M1 ve M2 yöntemleri arasında anlamlı bir korelasyon olmasına rağmen ($R^2 = 0,655$, $p < 0,01$), M2 yöntemi polen konsantrasyonu ile daha fazla ilişkiliydi. Polen ve DNA içeriği arasındaki korelasyon, polen mevsiminde gözlenen bölümler nedeniyle değişiklik göstermiştir. M2 yöntemi kullanılarak PCR verilerinden elde edilen DNA konsantrasyonları ile ışık mikroskobu ile belirlenen polen konsantrasyonları arasında hem II. Bölümde ($R^2 = 0,767$, $p < 0,01$), hem de tüm sezon boyunca ($R^2 = 0,469$, $p < 0,01$) önemli derecede ilişki bulunmuştur.

Sonuç: M2 yöntemi, Zonguldak ili atmosferinden elde edilen karışık örneklerdeki Poaceae polenlerinin doğru bir şekilde belirlemiştir. Kodlanmayan *trnT-F* cpDNA bölgesi ilk kez aerobiyolojik örneklerdeki Poaceae polenlerini belirlemek için kullanılmıştır. DNA özütlemesi gerektirmeyen bu metodun kullanımı ileride geliştirilecek eş zamanlı polen izleme cihazlarının önemli bir adım olabilir. Polen ve çoğaltılan DNA içeriği arasındaki ilişkinin kuvveti daha düşük emme kapasitesine sahip bir örnekleyici ve qPCR gibi daha hassas bir tekniğin kullanımıyla artırılabilir.

Anahtar Kelimeler: PZR; Polen teşhisi; *trnT-F* bölgesi; DNA miktarı; Poaceae.

Introduction

Several biological substances, such as pollen, mold spores, parts of plants and animals and bacteria and viruses, can be found in air sample preparations [1]. Pollen and spores have been monitored in different parts of world for a long time. Atmospheric pollen and spore monitoring are widely used for aware sensitive individuals. Several studies have shown

that the efficacy of immunotherapy increases, if it is started before the pollen season [2]. Pollen monitoring is also useful for estimating the start date for immunotherapy [3].

Methods to determine atmospheric content have been diversified throughout the last decade. Discriminating pollen and spores using a microscope is difficult and requires a trained staff. Therefore, there have been some attempts to determine pollen using image processing and molecular biological techniques [4]. Some studies have analysed allergens in samples collected with particle samplers using an enzyme-linked immunosorbent assay technique [5]. These studies have the advantage of directly determining substances that cause allergy using other than the traditional microscopic analysis. However, allergens are contained on some particles, such as starch granules, which are generally smaller than 1–2 μm and released from pollen easily by altering humidity changes [6]. Because pollen and particles have different aerodynamic properties, there is an imbalance between pollen and allergen concentrations in the atmosphere. In some cases allergen levels were observed when no pollen grains were detected in the atmosphere [7].

Molecular techniques based on the polymerase chain reaction (PCR) have also been used to identify pollen DNA in the air. Müller-Germann et al. quantified *Betula* pollen in a DNA analysis using specific primer pairs for single-copy gene (*BP8*) and a multi-copy gene (internal transcribed spacer [ITS]) [8]. They used qPCR and DNA sequence analyses and obtained a strong correlation with pollen data taken from the COSMO-ART model system. In a more recent study, *Juniperus* pollen was measured in 105 air samples during 2013–2015 with qPCR and species-specific primers and probes. The correlation between pollen and DNA content was very high ($R^2 = 0.902$) [9]. Variability in the ITS sequence has also been used to quantify DNA in mugwort and ragweed [10]. These techniques are also useful for determining long distance transported pollen, because pollen in the air can be identified at the species level, whereas the genus or family level is most commonly used in microscopic analyses. Using *matK*-specific primers, it was confirmed that some *Juniperus* pollen observed in the Tulsa atmosphere originated from *J. ashei* transported from South Oklahoma and Texas to Canada [11]. Leontidou et al. reported that the chloroplast *trnL* gene region can be used to identify pollen. That study differentiated *Juniperus* and Poaceae family samples with different complexities [12].

Although detection of pollen to the species level is appropriate for systematic purposes, scalability on detection of pollen in the atmosphere is more important to represent the allergenic load for sensitive individuals. Pollen allergens generally have conserved sequences. Therefore,

pollen of systematically related species often cross-react. Some individuals who are sensitised to pollen can show allergic reactions against some foods [13]. Because cross-reactivity is common among Poaceae pollen, grass pollen mix is generally used to diagnosis of allergy. Hence the region to be amplified should allow scalability of pollen identification either at the species and family levels.

The non-coding *trnT-F* cpDNA region includes three highly conserved tRNA genes. However, the spacers separating these genes are highly variable and help determine the relationships at the species and genus levels [14, 15]. The spacer region consists of several hundred base pairs. Other studies have used the *trnT-F* cpDNA region to investigate the phylogeny of some families and orders [16–18]. This region is also useful for identifying complex mixes of plants in complex mixes. Wallinger et al. used a *trnT-F* cpDNA specific primer to identify the plant species eaten by insect larvae [19].

In this study, we determined the correlation between the quantity of DNA amplified using *trnT-F* cpDNA specific primers and Poaceae pollen concentration. We also carried out optimisation of the DNA quantification method in the samples from a High Volume Air Sampler (HVAS), which contains higher amount of abiotic and biotic particles than those of traditional pollen and spore samplers.

Materials and methods

Collection of atmospheric samples and pollen materials

We analysed 40 daily air samples collected by a HVAS placed on the roof of a building in Zonguldak during 13 May–18 June in 2016. The sampler directs air that is suctioned in from the atmosphere (900 L/min) onto polyurethane filters placed in cascades. The sampler is equipped with two cascades. The upper cascade collects the particles which size of particulate matter (PM) is bigger than 10 μm , whereas the bottom cascade holds those of 10 μm > PM > 2.5 μm . The filters were changed daily at 12:00.

Poaceae pollen was collected using a Burkard 7-day pollen and spore trap. The trap was operated at 10 L/min. Daily pollen amount was counted in the entire full area and expressed as pollen/m³.

Phleum pratense L. and *Setaria viridis* (L.) P. Beauv. pollen from Zonguldak were collected and used as a positive control. *Betula pendula* Roth and *Ambrosia artemisiifolia* L. pollen which collected from Artvin and Düzce and an atmospheric sample that belongs to on the date of 20

February were used as negative controls. A small amount of pollen from both control groups was directly added to the PCR mix, according to manufacturer's instructions.

Extraction of filters and condensation of extracts

Only the upper filters (PM > 10 μm) were used in this study. The filters were cut into three equal parts before extraction. Two filter parts were extracted separately in ammonium-bicarbonate buffer (pH 8.1) and were aliquoted into 5 mL vials. Two different extraction methods (M1 and M2) were used for optimisation. Some of the vials was directly lyophilised (M1), whereas other vials were clarified by centrifugation at 6000 $\times g$ for 10 min before lyophilisation (M2).

PCR and DNA quantification

The PCR experiment was carried out using Plant Direct Kit (Thermo Scientific, Waltham, MA, USA). Before PCR, the vials were resuspended in phosphate buffered saline. A 1 μL sample was directly used as the DNA template. The *trnT-F* cpDNA region was amplified using a Poaceae specific primer pair [19] (Table 1). The PCR reactions were performed on the T100 thermal cycler (BioRad Laboratories, Hercules, CA, USA). The following program was used for the PCR reactions: after a 2 min initial denaturation at 94°C, 40 cycles of 20 s at 95°C, 1 min at 50.5°C and 1 min at 72°C, followed by 2 min at 72°C.

The amplified DNA fragments were quantified using the Qubit® dsDNA HS Assay Kit (Life Technologies, Foster City, CA, USA) on a Qubit® 2.0 Fluorometer (Life Technologies). Briefly, 1 μL of each sample was added to a microcentrifuge tube as part of the High Sensitivity DNA kit. After a 2 min incubation, the amount of DNA was measured. Agarose gel (2%) electrophoresis was used to confirm replication and verify a single amplified band.

Statistical analysis

Spearman's rank order test and a non-parametric correlation analysis were used to determine the relationship

Table 1: List of Poaceae-specific primers used in the polymerase chain reaction analysis.

| Primer name | Primer sequences (5'-3') |
|--------------|--------------------------|
| Poa-gen-S541 | GCTTTCTCATTCTACTCTTTTC |
| Poa-gen-A551 | CTTTTCTGTGCATCATCTAG |

between pollen and the quantity of DNA using R statistical programming language [20]. A p-value < 0.05 was considered significant.

Results

The total pollen index during the pollen season was 1442. The peak day of Poaceae pollen was 30 May (12.4 pollen/m³). Three distinct episodes were observed during the pollen season of 2016 in Zonguldak. The majority of the pollen was observed in episode II. A positive correlation was obtained separately for these episodes using both methods. The strength of the correlation both methods was higher than that of the total season (Table 2).

The specificity of the primer pairs was tested against the positive controls (*P. pratense*, *S. viridis*, 13 May and 21 May samples) and negative controls (*B. pendula*, *A. artemisiifolia* and 20 February sample) groups (Figure 1). The PCR results revealed one DNA band at 252 bp as the positive control.

The two preparation methods were also tested to optimise the results. The method in which the samples were clarified by centrifugation (M2) was the most effective. Although it varied depending on the method used for extraction, total DNA quantities were differed between 1.71×10^5 and 1.97×10^5 . Despite that a higher total DNA content was obtained with the M1 method and the two methods were correlated ($R^2 = 0.655$, $p < 0.01$), a positive correlation was only observed between pollen and DNA content with the M2 method ($R^2 = 0.469$, $p < 0.01$) (Table 2).

Discussion

It is a new challenge to determine atmospheric pollen content using molecular biological techniques. Several studies have identified and quantified pollen in atmospheric samples based on DNA content. Many gene regions have been used for this purpose. This is the first time that optimised PCR assays based on *trnT-F* cpDNA specific

primers have been used to identify Poaceae pollen DNA, were used by this study. Müller-Germann et al. analysed air filter samples by qPCR using two different primer pairs (*BP8* and *ITS*). They reported a high correlation coefficient ($R^2 = 0.92$) between the quantity of DNA and pollen levels of the COSMO-ART model system [8]. In another study, *matK* specific primers were used to identify *Juniperus* pollen in 105 air samples collected with a Burkard spore trap. A significant correlation was found between pollen concentrations determined by qPCR and those collected with the Burkard pollen and spore trap ($R^2 = 0.902$) [9]. They used a Hirst type pollen sampler, which is similar to the Burkard. The correlation between pollen and quantity of DNA was also high, as in other studies [12].

We obtained a relatively lower correlation than these studies, which may be related to using different samplers for collecting pollen and DNA containing particles. Mohanty et al. used samples from a single sampler for both microscopic and DNA analyses [9]. The sampler used to perform the DNA analysis has been used for the pollen

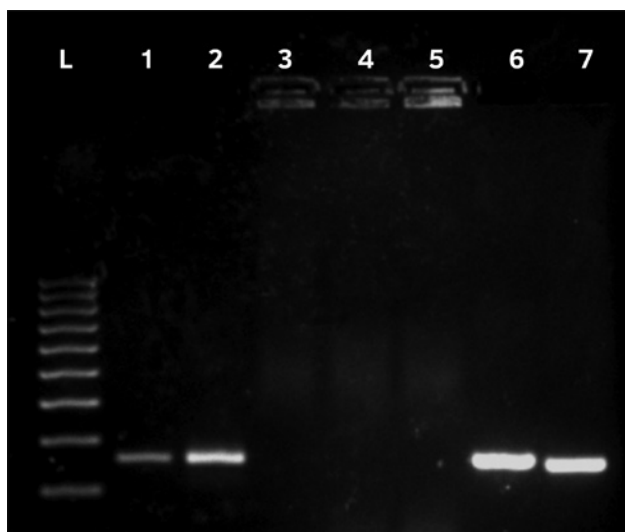


Figure 1: Agarose gel view of the control groups. (L) DNA ladder (100–1000 bp), (1) 13 May sample, (2) 21 May sample, (3) 20 February sample, (4) *A. artemisiifolia* pollen, (5) *B. pendula* pollen, (6) *S. viridis* pollen, (7) *P. pratense* pollen.

Table 2: Results of the correlation analysis between pollen and quantity of DNA obtained by two methods.

| | Pollen level (pollen grains/m ³) | M1 | | M2 | |
|-----------------------------|--|--------------------|--------------------|--------------------|--------------------|
| | | Corr. | DNA content | Corr. | DNA content |
| Total | 98.8 | 0.122 | 1.97×10^5 | 0.469 ^b | 1.71×10^5 |
| Episode I (13–17 May) | 17.7 | –0.7 | 9.46×10^3 | –0.1 | 1.23×10^4 |
| Episode II (20 May–06 June) | 55.4 | 0.491 ^a | 4.67×10^4 | 0.767 ^b | 6.71×10^4 |
| Episode III (13–20 June) | 25.7 | 0.083 | 5.83×10^4 | 0.598 | 6.53×10^4 |

^aCorrelation is significant at the 0.05 level (2-tailed). ^bCorrelation is significant at the 0.01 level (2-tailed).

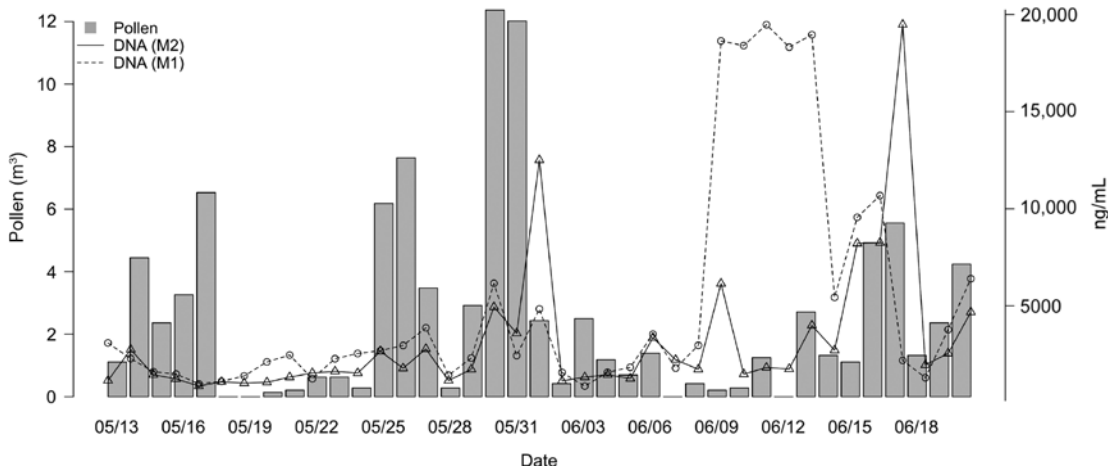


Figure 2: Changes in Poaceae pollen and DNA content amplified using a Poaceae-specific primer pair on filters containing the size of PM is bigger than 10 μm .

count in many studies [21, 22]. We used a fluorometer to quantify the DNA different from other studies of which were used qPCR which allow more accurately DNA quantification. This could be another reason why a lower correlation was observed between Poaceae pollen and the quantity of DNA in this study. According to correlation results of the two methods, centrifugation has positive effect on the correlation between pollen concentration and the quantity of DNA in the M2 method (Figure 2). Hence, the higher particulate content in the air samples due to the high suction rate of the sampler may have decreased the strength of the correlation (Table 2).

Pollen season in Zonguldak appears not to be homogenous. We obtained comparable results from other studies at the level of episodes using both methods. In particular, high DNA content was obtained by the two methods during episode III. DNA extraction from plants is harder than that from other biological samples because of the cell wall. However, pollen has an opening on the surface called the aperture. The aperture plays a key role in pollen tube germination and allows the release of pollen by a simple force, such as centrifugation. Hence, centrifugation, as part of the M2 method, could have helped extract DNA from the pollen, whereas the M1 method may have increased DNA content by including other Poaceae plant parts sucked by the sampler. The highest concordance ($R^2=0.767$, $p<0.01$) between the quantity of DNA and pollen count was observed in episode II with the M2 method.

On the other hand, lower DNA content than pollen concentration during episode I may be an indicator of pollen transportation. As mentioned above, pollen is easily released into the air by changes in humidity level. Therefore, pollen captured by the Burkard sampler in episode I may have already released DNA during transport.

Although identifying pollen at the species level can be useful to demonstrate long distance pollen transport, it does not represent the allergenic load to sensitive patients. The majority of pollen allergens have conserved sequences, so polysensitisation to systematically related pollen is very common in a sensitive patient [23]. Thus, scalability in determining DNA-based pollen should be a top priority. We cannot describe a Poaceae allergy by looking at a single *Lolium perenne* L. or *P. pratense* pollen grain in the air. We should look at the genus or family level according to necessity. The *trnT-F* cpDNA region was useful in the present study to identify Poaceae pollen. The next generation sequencing method, such as metabarcoding, has been used to identify pollen at the species and family levels [21, 22]. However, the next-generation sequencing is expensive and time consuming.

Another advantage of our method is that it is not a necessary step of DNA extraction due to the kit used for DNA amplification. On the other hand, a sampler such as the Burkard, which is changed weekly, may not be suitable for real-time monitoring. Impactor samplers with multi-vials may be appropriate for this purpose. The use of a method that does not require DNA extraction may be a crucial step toward real-time pollen monitoring devices developed in the future.

As a result, it was shown here that the *trnT-F* cpDNA intergenic spacer region is very useful for quantifying atmospheric Poaceae pollen DNA at the family level. The necessity of the centrifugation step in the present method depends on whether the content of the other particles is higher than the pollen due to the sampler absorption rate. Thus, the use of different samplers, such as a low-volume air sampler, and a more sensitive quantifying technique, such as qPCR, will allow for the direct determination of

atmospheric pollen faster than any other method in the literature and will be free from this requirement.

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