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qPCR analysis of *Puccinia* spp. in turfgrass areas in Türkiye

Türkiye'deki çim alanlarında *Puccinia* spp.nin qPCR analizi

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ABSTRACT

In recent years, with the increase in parks, gardens, recreation areas, picnic areas and refuges with urbanization, the interest in turfgrass areas that beautify the aesthetic appearance of these areas has also increased. In these areas, rust diseases are also becoming increasingly common and causing problems. Rust diseases are an important pathogen group that needs to be monitored due to their ability to create new races and their airborne transmission. In this study, surveys were conducted in turfgrass areas in 8 provinces of Türkiye and 110 turfgrass leaf samples showing rust symptoms were collected. As a result of DNA isolation and qPCR analysis from pustules on the leaf surface, 37 *Puccinia coronata*, 32 *P. graminis*, 18 *P. striiformis* and 23 *Puccinia* spp. have been detected. It was determined that the most common rust species that causes disease in turfgrass areas in Türkiye is *P. coronata*. This species was followed by *P. graminis* and *P. striiformis*, respectively. While *P. striiformis* was mostly isolated from Kayseri and Istanbul provinces, *P. coronata* and *P. graminis* were mostly isolated from İstanbul.

INTRODUCTION

Puccinia genus is a group of fungi that includes airborne and obligate pathogens species that cause problems in turfgrass areas. The agents that cause rust diseases in turfgrass damage the leaves due to the pustules they form on turfgrass leaves, seriously deteriorate the quality of the turfgrass and can cause great economic losses. The increase in rust diseases is closely related to temperature and humidity. When the disease first begins, yellow-orange urediospore pustules appear on the leaves, which causes necrosis on the leaves in later periods. In time, the pustules spreads on the leaves and absorbs nutrients in plant cells, destroys chlorophyll and prevents the plant from performing photosynthesis and respiration, which negatively affects the physiology and general health of the plant. All turfgrass varieties are sensitive to the disease (Smiley et al. 2005), and it has been determined that it can be seen in all regions of Türkiye when suitable conditions for disease development occur.

There are currently a total of 39 different species of rust fungi (*Puccinia, Physopella* and *Uromyces*) known to be pathogenic to turfgrasses (Smiley et al. 2005). But *Puccinia coronata* Corda (crown rust), *Puccinia graminis* Pers (stem rust),

Puccinia striiformis Westend (stripe rust), *Puccinia recondita* Roberge ex Desmaz. and *Puccinia brachypodii* G. Otth (leaf rusts) are considered the most prevalent rust pathogens of turfgrass (Karakkat et al. 2018, Smiley et al. 2005).

While intermediate hosts, spore structures on intermediate and main hosts, their biology of rust diseases other than turfgrass plants have been examined in more detail, less information is available on turfgrass rusts than on rust diseases in other cereals. To develop different methods of control against a pathogen, such as resistant varieties, all morphological forms and the biology of the agent must be well known. Since intermediate hosts come into play in the biology of rusts, it is difficult to follow them. Additionally, since there are so many different *Puccinia* species, it is difficult to distinguish their spore differences visually and the risk of making mistakes is high. Before the development of molecular techniques, studies such as effective control strategies and the development of resistant varieties in turfgrass rust diseases were difficult due to the insufficient information available about these factors. Given the enormous economic and aesthetic problems associated with turfgrass rust diseases and the confusion in accurately identifying and classifying these pathogens, improving turfgrass breeding programs and expanding knowledge about the turfgrass rust system is an important priority. Evaluating these fungi at the molecular level, developing an accurate identification procedure, and implementing an effective inoculation protocol to maintain pathogen cultures will improve the ability to study turfgrass rust fungi.

It is reported that if rust diseases are not controlled, they negatively affect turfgrass seed production and this causes significant losses in the seed production industry. Studies report that rust diseases reduce seed yield by 25% - 98% (Beirn et al. 2011, Pfender 2009). Since rusts are diseases that can spread very quickly with the wind and constantly create new races, it has become important to monitor them regularly and develop fast and accurate diagnostic methods take new precautions and develop strategies. In addition, since the sensitivity of different fungi to fungicides varies, correct identification of the fungus is essential for effective control of the disease. Classical diagnosis of *Puccinia* species is usually made based on the shape, size and color of their spores under the microscope (Zhang et al. 2022). However, the fastest and most accurate diagnosis is important in distinguishing the species. Molecular analysis methods are considered the most reliable and fastest method for rust fungi (Stackhouse et al. 2020). Quantitative real-time polymerase chain reaction (qPCR) is a rapid, accurate, and highly sensitive molecular detection method widely used to quantify pathogenic fungi, viruses and bacteria, and fungi from a wide variety of hosts (Mirmajlessi et al. 2015, Stackhouse et al. 2020). Several PCR-based molecular assays have been developed for turfgrass pathogens, including SYBR green probes to identify *Ophiosphaerella agrostis* in *Agrostis stolonifera* (creeping bentgrass), a set of real-time qPCR assays for the detection of Puccinia species causing turfgrass rust diseases, and a TaqMan assay to detect *Magnaporthiopsis* poae in Kentucky bluegrass (Beirn et al. 2011, Kaminski et al. 2005, Zhao et al. 2012). This study is the first comprehensive rust survey and detection study conducted on turfgrass areas in Türkiye.

MATERIALS AND METHODS

Survey

In the survey studies in 2015, leaf samples with rust symptoms were collected from parks and gardens, recreation areas, and refuges in İstanbul, Ankara, Kayseri, Bursa, Antalya, İzmir, Aydın and Muğla provinces. Depending on the size of the controlled land, in the surveys made to represent that area, 5 samples up to 5000 m2, 10 samples between 5000-10000 m2, 15 samples up to 10000 m2-15000 m2, 20 samples from areas larger than 15000 m2 were collected (Aktaş 2001). The collected leaves were placed in ice boxes and brought to the laboratory for molecular identification.

Real-time PCR analysis

For molecular identification, urediniospores were carefully scraped on turfgrass leaves and transferred into 1.5 ml microtubes, DNAs were extracted using DNeasy Plant Mini Kit (Qiagen, USA). Real-timePCR studies were carried out using different specific primers and probes (Table 1) (Beirn et al. 2011) with genomic DNA obtained from plants with rust symptoms and rust types were identified. Roche LightCycler 480 II Real time-PCR device was used in the study and the raw data were analyzed with the "Absolute Quantification/Second Derivative Maximum" method in Lightcycler 1.5 software and the results were obtained. In addition, the raw data were examined with the "Fit point" method and the effects of Background, log phase and plateau phase on CP (Crossing Point) values were evaluated.

In real-timePCR studies, the Roche Lightcycler® Probes Master ready mix was used, taking into account the optimum values specified in the kit manual. In a preliminary study, the suitability of the device for "ramp rate (°C/s)" and waiting times at relevant temperatures were tested for a total of 10-20 ng template DNA and 20 µl reaction volume. In addition, the optimum binding temperature was 60 oC and the two-step (denaturation and binding/extension) replication step was found to be more efficient and accordingly; PCR reaction for each sample; 10 µl 2x Roche Probe Master Mix, 2 µl primer/probe mix (containing 10 mM primer 1, 10 mM primer 2 and 10 mM probe), 3.0 µl molecular biological purity water, 5 µl template DNA (10-20 ng) It was carried out in a total reaction volume of 20 µl. Real-time PCR conditions are; initial denaturation 95 °C 10 min, denaturation 95 °C 15 sec, binding/extension 60 °C 60

Table 1. Polymerase chain reaction primers and hydrolysis probes used in this study

Primers and Probes	
FrITS2Cr (Puccinia coronata forward ITS2);	3'-TTTGTGGATGTTGAGTGTTGC-5'
RrITS2Cr (P. coronata reverse ITS2);	3'-TCCCACCTGATTTGAGGTCT-5'
FrITS1Pu (P. graminis and P. striiformis forward ITS1);	3'-CCTGCGGAAGGATCATTATT-5'
RrITS1Pu (P. graminis and P. striiformis reverse ITS1);	3'-TTTGGTTACATTCATTTAAACTTGTG-5'
PuSTM-ITS1 (Puccinia graminis probe);	3'-Cy3-TTAGAGTGCACTTTATTGTGGCTCAACTCTCT-BHQ1-5'
PuSTR-ITS1 (Puccinia striiformis probe);	3'-FAMCGTAACTTCTTTATTGAATGTTGCATTACCCTCCC-BHQ1-5'
PuCR-ITS2 (Puccinia coronata probe);	3'-FAM-TACTTGCCATCTTTTGAAAGGAGGGA-BHQ1-5'

sec, cooling 40 °C 5 min.

RESULTS AND DISCUSSION

As a result of the survey studies, 110 leaf samples showing rust symptoms were taken from grass areas in 8 provinces. In field observations, it was observed that the rust disease started as spots ranging from light green to yellow on the leaves, leaf sheaths and stems, and advanced symptoms turned into regular or scattered yellow, orange or brick red pustules, depending on the type of rust (Figure 1).

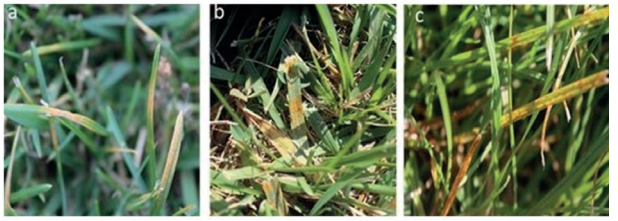

Figure 1. Symptoms of *Puccinia striiformis* (a), *Puccinia graminis* (b) and *Puccinia coronata* (c) on turfgrass leaves in the survey areas

Puccinia spp. were identified by real-time PCR studies using different specific primers and probes. As a result of the identification studies, 37 *P. coronata*, 32 *P. graminis* and 18 *P. striiformis* species were identified. No results were obtained from 23 samples with the primers and probes used. It was concluded that these 23 rust species belong to species other than the three species. Rust species were seen in all regions, but *P. striiformis* was more common in Kayseri and İstanbul provinces (Table 2). *P. coronata* and *P. graminis* were mostly isolated from İstanbul.

As a result of the real-time PCR study, the most common rust species that cause disease in turfgrass areas in Türkiye was determined to be *Puccinia coronata*. This species was followed by *P. graminis* and *P. striiformis*, respectively. The frequency of *P. coronata, P. graminis* and *P. striiformis* isolation was 33.63%, 29.09% and 16.36%, respectively. It was concluded that 23 rust species belonged to the other rust species that were not included in the three species, as the primers and probes used for the three species did not give the desired curves (Figure 2). Among the *Puccinia* spp. in turfgrass areas, only these three species can be identified molecularly (Stackhouse et al. 2020). Studies should be carried out to diagnose other species of rust using molecular methods, and effective primers and probes need to be developed. In this regard, a study was conducted in the USA, 66 rust-infected turfgrass samples were collected from different regions of the USA and these collected isolates were evaluated by qPCR study using primers and probes developed from the ITS-5.8 rDNA region. Analysis results showed that 67% of the samples were P. coronata, 28% were *P. graminis* and 5% were *P. striiformis* (Beirn et al. 2011). In this study, the most isolated species was P. coronata and all three rust species were detected in all provinces of Türkiye. *P. graminis* and *P. striiformis* are also common rust species in wheat production areas in Türkiye (Aktaş 2001).

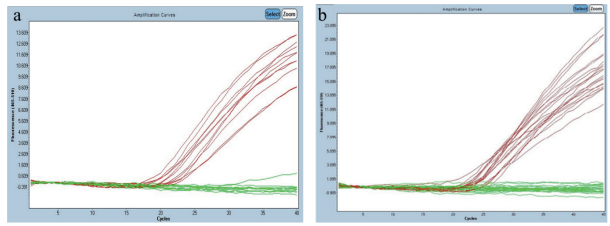

Figure 2. Amplification curves of some samples obtained in real-time PCR with *Puccinia graminis* (a) and *Puccinia striiformis* (b) specific primers and probes (red curves refer to positive samples, green curves refer to negative samples and the template belongs to the control without DNA

Most turfgrass varieties used in the turfgrass areas in Türkiye are sensitive to rust diseases (Smiley et al. 2005), and it has been determined that rust diseases can be seen in all regions of Türkiye when suitable conditions for disease development occur. Rust fungi, located in the Pucciniales order of the Basidiomycetes class, constitute the largest group of obligate pathogenic fungi. It is estimated that there are more than 7000 species of rust in the world, and only 3/4 of them have been fully identified (Hawksworth et al.

Table 2. The number of samples taken from the provinces, determined rust species, numbers and percent occurrence rates of rust species

Provinces	Number of leaf samples with rust symptoms taken	*P. coronata*	*P. graminis*	*P. striiformis*	Other rust species
İstanbul	26	10	7	4	5
Ankara	19	8	5	1	5
Bursa	18	6	6	2	4
Kayseri	18	5	4	7	2
İzmir	10	4	3	1	2
Aydın	7	2	3	1	1
Muğla	6	1	2	1	2
Antalya	6	1	2	1	2
	110	37	32	18	23
Percent occurrence rates (%)		33.63	29.09	16.36	20.90

1995). The largest genus, *Puccinia,* contains approximately 4000 species, nearly 650 of which are pathogenic to various plants within the Poaceae family (Abbasi 1996). There are currently a total of 39 different species of rust fungi (belonging to *Puccinia, Physopella* and *Uromyces* genus) known to be pathogenic to turfgrass but 10 of these commonly cause infection in turfgrasses (Smiley et al. 2005). In another study, 56 *Puccinia* species and 14 *Uromyces* species were associated with the invasion of the Poaceae family, which includes grass and non-grass hosts (Afshan 2008). Considering the complications of morphology-based rust taxonomy, the use of molecular sequence data in conjunction with morphology has become a powerful tool for the study of these fungi at the species level (Aime et al. 2006, Beirn et al. 2011, Maier et al. 2007, Van der Merwe et al. 2007). In addition, there are many different races of rust fungi and the disease severity of these races on different turfgrass varieties varies. Some turfgrass varieties may become susceptible to some types of rust because they can create new strains over time that can also infect resistant varieties (Avasthi et al. 2023). For this reason, the detection and monitoring of rust species in turfgrass areas is important.

In a study conducted in the Midwestern United States, rust species in turfgrass areas were monitored and determined via real-time PCR between 2013 and 2015. In the study, the most isolated species from Kentucky bluegrass was *P. graminis* with a rate of 69%. This was followed by *P. coronata* with a rate of 17%. Both species of rust were detected in 13% of the samples taken. In the same study, the population of 2 rust species was monitored in the region for 3 years. Both year and location effects were observed in population distribution. Additionally, variability was observed in pathogen-host relationships in the following years. It has been revealed that rust species differ between locations in the same year, and data taken from the same location for several years also show differences in terms of rust diseases distribution and density. For this reason, the density and distribution of rust species in turfgrass areas should be monitored at regular intervals. Watkins and Gaussoin (1992) stated that the rust disease caused by *Puccinia graminis* in turgrasses is commonly seen in *Poa pratensis* (fescue grass), *Lolium perenne* (English ryegrass), *Festuca arundinaceae* (reedy fescue) and *Zoysia japonica* (Japanese ryegrass). They also stated that cool climate turfgrass plants (*P. pratensis, L. perenne, F. arundinaceae*) are damaged more severely in the summer (Smiley et al. 2005). There is currently no licensed commercial fungicide for conrol against rust diseases in turfgrass areas in Türkiye. In the future, studies should be carried out to control rust diseases in turfgrass.

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Author's Contributions

Authors declare the contribution of the authors is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

ÖZET

Son yıllarda şehirleşmeyle birlikte park, bahçe, rekreasyon alanları, piknik alanları ve refüjlerin artmasıyla birlikte bu alanların estetik görünümünü güzelleştiren çim alanlarına ilgi de artmıştır. Bu alanlarda pas hastalıkları da giderek yaygınlaşmakta ve sorun oluşturmaktadır. Pas hastalıkları, yeni ırk oluşturma kapasiteleri ve hava yoluyla taşınmaları sebebiyle takip edilmesi gereken

önemli bir patojen grubudur. Bu çalışmada, Türkiye'nin 8 ilindeki çim alanlarında sürveyler düzenlenerek, 110 adet pas belirtisi gösteren çim yaprak örneği toplanmıştır. Yaprak yüzeyindeki püstüllerden DNA izolasyonu ve qPCR analizi sonucunda 37 adet *Puccinia coronata*, 32 adet *P. graminis,* 18 adet *P. striiformis* ve 23 adet *Puccinia* spp. tespit edilmiştir. Türkiye'de çim alanlarında hastalığa neden olan en yaygın pas türünün *P. coronata* olduğu saptanmıştır. Bu türü sırasıyla *P. graminis* ve *P. striiformis* takip etmiştir. *P. striiformis* en fazla Kayseri ve İstanbul ilinden izole edilirken, *P. coronata* ve *P. graminis* en fazla İstanbul ilinden izole edilmiştir.

Anahtar kelimeler: çim, pas, real-time PCR, Türkiye

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