# Detection of *Erysiphe necator*, the Causal Agent of Powdery Mildew on Grapevine, and Determination of their Mating Types in Southern Syria Using Some Molecular Markers

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## Abstract

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Powdery mildew caused by *Erysiphe necator*, is one of the most economically damaging diseases of grapevine throughout the world. This study aimed to identify the mating types of this pathogen in Syria using molecular markers. Three DNA extraction methods: Promega Kit, phenol chloroform isoamyl alcohol (PCI) and SDS were compared. The amount and quality of DNAs obtained by the SDS method were suitable for PCR amplification and other molecular assays. PCR amplification using specific primers (Uncin 144 and Uncin 511) was performed and the expected amplicon of 300-400 bp was obtained from 29 isolates of *E. necator* collected from different geographical locations and from different grapevine cultivars. Results obtained showed that *E. necator* may cause atypical symptoms similar to those of downy mildew, in some grapevine cultivars according to the environmental conditions and training system used. Isolates of *E. necator* were classified into four groups according to geographical locations and grapevine cultivar, based on the observed variation in banding pattern with E07 primer and the constructed phenogram by using UPGMA. RAPD analysis of 39 *E. necator* isolates using the primer E07 showed the presence of two mating types, and the frequency of each varied depending on the geographical location, with a predominance of the - mating type which did not produce a 1000 bp band using the PCR primer E07.

**Keywords:** Powdery mildew, *Erysiphe necator*, RAPD, mating type, DNA extraction.

## Introduction

Grapevine (*Vitis vinifera* L.) is one of the world's oldest and most important fruit crops, nutritionally and economically. However, many important grapevine varieties are susceptible to powdery mildew, caused by *Erysiphe necator* Schw. [syn. *Uncinula necator* (Schw.) Burr.] (Ascomycota, Leotiomycetes, Erysiphales). This disease occurs in almost all countries and regions where grapes are grown and causes major loss of grape production each year (Akkurt *et al*., 2006), and it is the most economically significant disease of grapevines in southern Syria (Unpublished data).

Both asexual and sexual stages were reported for *E. necator*. Sexual reproduction results in the formation of chasmothecia (formerly Known as cleistothecia), when the colonies of two opposite mating types meet on the infected plant parts, and the temperatures are favorable for their development (Sawant *et al*., 2017). Chasmothecia can form on all infected tissues from early summer to autumn. Environmental factors such as temperature, day length, humidity, leaf age and host resistance do not affect chasmothecia initiation and, once initiated, only temperature and host resistance affect their growth (Legler *et al*., 2013; Pearson & Gadoury, 1987). In Syria, chasmothecia were observed in 45.5% of studied vineyards (Alimad *et al*., 2016). *E. necator* has been reported to overwinter as mycelium in dormant buds and/or as chasmothecia on the bark of vines or in the soil (Cortesi *et al.,* 2005; Miazzi *et al.,*2003). In southern Syria, *E. necator* survived as mycelium in dormant grapevine buds during the winter season, and the ascospores did not have any role in the initiation of spring infection (Alimad *et al*., 2017).

Little information is available about the genotypic diversity of *E. necator*, due to its obligate biotrophic nature, and the fungus cannot be grown on artificial media (Brewer *et al*., 2011; Miazzi *et al*., 2003). In fact, few molecular studies were conducted on mildew fungi, with the exception of the highly sporulating powdery mildew of cereals, *Blumeria graminis* (formerly named *Erysiphe graminis*), where conidia can be easily collected from host leaves by tapping or blowing (Gadoury *et al*., 2012; Newton *et al.,* 2004; Yousefi *et al*., 2010). Morphologically similar but genetically distinct groups have been identified in Australian and European populations of *E. necator* (Evans *et al.,*1997; Miazzi *et al.,* 2003; Péros *et al.,* 2005; Stummer *et al.,* 2000). RAPD technique proved to be very useful for studying genetic variation in a number of fungal species including the biotrophic fungi that cause powdery mildew and wheat leaf rust (Delye *et al.,* 1997; Newton *et al.,* 2004; Yousefi *et al*., 2010). Genetic diversity has been evaluated in *E. necator* using different techniques, such as transposon-PCR (Bouscaut and Corio-Costet, 2007), ISSR (Cortesi *et al.,* 2005), nested allele-specific (NAS) PCR (Delye and Corio-Costet, 1998) and SCAR primers (Hajjeh *et al.,* 2005). Polymerase chain reaction (PCR) was used to distinguish *E. necator* isolates from other species of powdery mildew using

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a pair of primers Uncin144 and Uncin511 (Falacy, 2003). Péros *et al*. (2005) described two genetically distinct groups of *E. necator* in southern France. Group A was characterized by low genotypic diversity and only one mating type. Group B isolates were more diverse, and both mating types were present. Whereas, Yousefi *et al*. (2010) classified 20 isolates of *E. necator* in seven distinct groups in Iran using RAPD technique. This is the first study on the detection of *E. necator* and determination of its mating types in Syria using molecular markers.

## Materials and Methods

**Sampling**

Samples were collected in 2016 according to Montarry and Cartolaro (2009), at the beginning of the season (the beginning of May), and later in mid-June. Samples consisted of leaves showing typical initial symptoms of powdery mildew characterized by small white to grey powdery spots of less than 0.5 cm in diameters. Leaves of Black cultivar (Kanawat 2 site) showing symptoms similar to those caused by downy mildew, pale to yellowish spots at the upper surface, with or without grayish–violet powdery growth at the underside of the leaves were also collected. Each leaf spot on the same plant was collected independently and considered as an isolate. Twenty-nine samples (isolates) were obtained from five different sites (Table 1). Spots were cut, transferred to Eppendorf tubes and stored at -20℃ until use. Another ten samples were collected at the end of the season (November) from five vineyards at Albassa and Almarj sites, where leaves were completely covered by mycelium and conidia (Table 1). Each sample (leaf) was stored in a Petri dish (9 cm) at -20℃ until use.

***Erysiphe necator* DNA Extraction**

Three DNA extraction methods were compared to identify the most appropriate DNA extraction procedure that can produce good quantity and quality of fungal DNA.

**(1) DNA extraction using Promega DNA isolation Kit -** samples (spots and leaves covered with mycelium and conidia) were ground with liquid nitrogen to a fine powder in a pre-cooled, sterilized mortar, then transferred to Eppendorf tubes. Manufacturer's instructions for DNA extraction were followed according to the kit technical manual.

**(2) Phenol: Chloroform: Isoamyl Alcohol (PCI) method -** 1 g of sucrose was added to 50 ml of extraction buffer without SDS, and heated at 65℃ until fully dissolved. 2% (1 ml/50ml) mercaptoethanol and 2% PVP were added. Samples (leaves with mycelium and conidia) were ground in the previous extraction solution using a sterilized mortar, transferred to 1.5 ml tubes, incubated at 42℃ for 30 min, centrifuged for 10 min at 10000 rpm, suspended in 1 ml buffer, incubated in a water bath for 60 min at 37℃. Then, 500 µl Phenol: Chloroform: Isoamyl Alcohol (24:24:1 v) were added, mixed for 10 min, centrifuged for 10 min at 10000 rpm. The water phase was transferred to a clean 2 ml reaction tube. DNA was precipitated by adding 350µl ice-cold isopropanol, placed at -20℃ for 30 min, centrifuged for 10 min at 10000 rpm. Pellet was washed with 300µl 70% ethanol, centrifuged for 10 min at 10000 rpm. Supernatant was removed, and pellet was air-dried for 15 min, dissolved in 50 µl TE (10 mMTris-HCl, 1mM EDTA), 2 µl RNase (10 mg/ml) were added, and incubated for 30 min at 37℃. Then, DNA was diluted to 40 ng/µl.

**(3) DNA extraction SDS method after washing in ethanol -** 1 ml of ethanol 99% was added to 2 ml Eppendorf tubes, each containing and infected plant disc, and 3 ml of ethanol were added to 9 cm petri dishes containing full vine leaves (one of each). Samples were soaked for two hours, then plant pieces were removed, and the liquid was transferred to 2 ml Eppendorf tubes, centrifuged for 10 min at 10000 rpm. The pellet was suspended in 1 ml buffer (0.1M Tris-HCl, pH=8.2, 50 mM EDTA, 0.1M NaCl, 2% SDS, 1 mg/ml proteinase K), incubated in a water bath for 60 min at 37℃. The remaining steps followed were as described in the previous method.

**DNA Quantity and Quality Determination**

The quantity of the extracted gDNA was determined by measuring the UV absorbance at 260 and 280 nm using Power WaveX$^{TM}$ (BIO-TEK Instruments, Inc.) spectrophotometer. The quality of extracted gDNA was determined by gel electrophoresis on 0.8% agarose.

**Table 1.** Geographical locations and date of powdery mildew infected samples collected from different grapevine cultivars at different locations in Syria.

| Samples number (Isolate) * | Location | Cultivar | Collection date |
|---|---|---|---|
| 1 – 4 | DaherAljabel (Research center)/3 (RC/3) | Black | 15/6/2017 |
| 5 – 8 | DaherAljabel (Research center)/1 (RC/1) | Black | 15/6/2017 |
| 9 – 16 | Kanwat 2 (K/2) | Black | 2/5/2017 |
| 17 | Kanawat 1 (K/1) | Black | 2/5/2017 |
| 18 – 21 | DaherAljabel (Research center)/2 (RC/2) | Balady | 15/6/2017 |
| 22 – 28 | Kanwat 2 (K/2) | Balady | 2/5/2017 |
| 29 | Kanwat 1 (K/1) | Balady | 2/5/2017 |
| 30 – 34 | Albassa | Balady | 2/11/2017 |
| 35 – 39 | Almarj | Balady | 2/11/2017 |

***** Samples 1–29 = leaves showed initial powdery mildew symptoms; samples 30–39 = leaves were completely covered with mycelium and conidia.

**Molecular Identification of *E. necator* by Species-Specific PCR Assay**

The PCR was performed using species-specific primers (forward primer Uncin144 CCGCCAGAGACCTCATCC AA and reverse primer Uncin511 TGGCTGATCACGAG CGTCAC) according to Lawyer *et al*. (1993) with some modifications. The PCR reactions were performed in a 25µl reaction volume using 2X Master Mix. The PCR reaction contained 1.5 µl of each primer (10 mM), 12.5 µl Master Mix, 8.5 µl distilled water and 40 ng/µl DNA of each sample. Amplification was performed in an Eppendorf Master Cycler (Eppendorf, Hamburg). The PCR cycling conditions consisted of an initial denaturation step of 94°C for 5 min and subjected to 40 cycles of the following program 94°C for 30 s, 57°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. Twenty µl of each reaction mix and a 1Kbp DNA ladder (Fermentas, Germany) were loaded into a 2% agarose gel with TBE buffer (10X TBE buffer = 108 g Tris borate + 55 g Boric acid + 9.2 g EDTA, pH 8.0). After 2.0 hours at 100 V, the gel was stained with ethidium bromide and photographed.

**RAPD Analysis for Mating Type Determination and Genetic Diversity Assessment**

E07 primer (AGATGCAGCC) was used for mating type detection of 39 isolates of *E. necator*. The basis for considering isolates as (+ or –) was the amplification of a 1000 bp fragment using E07 primer (Yousefi *et al*., 2010). The reaction consisted of 10 µl of PCR Master Kit containing all PCR reaction compounds, with 3 µl of the primer, and 40 ng / µl DNA. Amplified products were analyzed using 2% agarose gel electrophoresis, and visualized under a UV transilluminator. For genetic diversity, a phenogram was constructed using UPGMA method to show relationships between isolates.

## Results and Discussion

**DNA Extraction and Molecular Identification of *E. necator***

Because obligate parasites cannot be grown in artificial media, the genomic DNA of *E. necator* was extracted from infected grapevine leaves with three different methods as explained in material and method section. Results obtained (Figure 1) showed that no DNA was extracted using the commercial kit from Promega. This may be due to the presence of phenolic compounds in the plant tissues that inhibited DNA extraction. Method 2 (chloroform: isoamyl alcohol) for DNA extraction after removing the phenolic compounds, allowed the DNA extraction from plant tissues, but not from fungal material. Although bands were seen on the 0.8 agarose gel (Figure 1), but no PCR amplifications were obtained using the specific primers for *E. necator*.

The amount and quality of DNAs obtained by the extraction method with SDS were suitable for PCR amplification and other molecular assays. The DNAs were then utilized for various PCR based analyses. PCR amplification using specific primers (Uncin144 and Uncin511) was performed and an expected band size of 300–400 bp was obtained from 29 isolates of *E. necator* (Figure 2). Primers pair Uncin144/Uncin511 amplified 367 bp fragment of *E. necator* DNA (Falacy *et al*., 2007). In fact, previous studies have showed a high specificity of this pair of primers in detection of *E. necator*, whereas it did not give any results in the detection of any of 35 other species causing powdery mildew on 46 different plant hosts (Falacy, 2003). Our results confirmed that all isolates collected from different geographic locations and from different vine cultivars were *E. necator*. Search in NCBI-Blast 2 (Altschul *et al*., 1997) based on nucleotide sequences of the primer pair used in this study assumed to be 368 bp.

Similar results were obtained when the DNA was extracted from full vine leaves covered by mycelium and conidia, where the expected band of 300 – 400 bp was obtained as shown in figure 3.

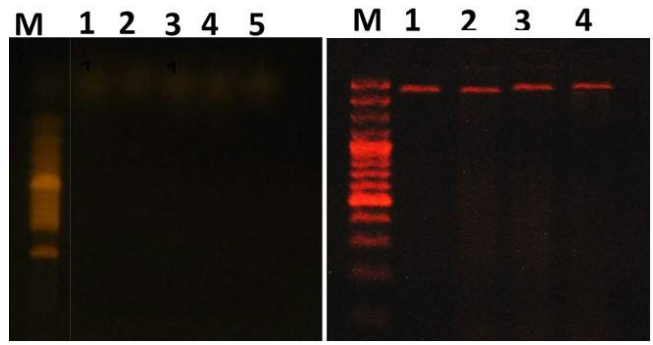


**Figure 1.** Ethidium bromide-stained 0.8 % agarose gel of the extracted DNA from infected leaves by *E. necator*. (Left) using Promega DNA isolation Kit; (Right) using DNA extraction method after removing the phenolic compounds. Lane M is a DNA ladder marker, and the remaining lanes are samples numbers.

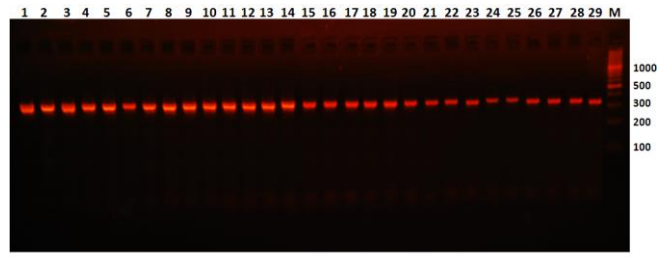


**Figure 2.** A band of 300–400 bp on 2% agarose gel after PCR amplification of DNA was obtained from 29 isolates of *E. necator* using the primers pair Uncin511/Uncin144. Samples 1–4 are of Black grape cultivar from Daher Aljabel (Research center)/3; Samples 5–8 of Black cultivar from Daher Aljabel (Research center)/1; samples 9–16 of Black cultivar from Kanawat 2; sample 17 of Black cultivar from Kanawat 1; samples 18–21 of Balady cultivar from Daher Aljabel (Research center)/2; samples 22–28 of Balady cultivar from Kanawat 2; sample 29 of Balady cultivar from Kanawat 1

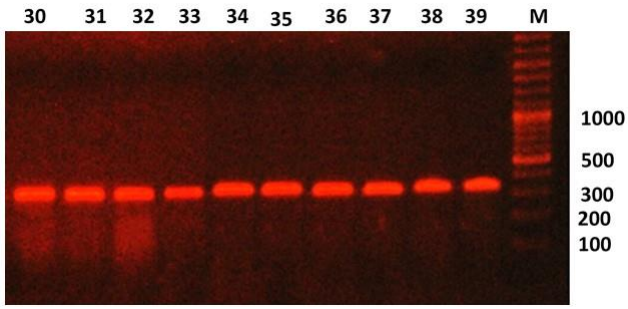


**Figure 3.** A band of 300–400 bp on 2% agarose gel after PCR amplification of DNA from 10 samples of full grapevine leaves covered with *E. necator* mycelium and conidia using a pair of primers Uncin511/Uncin144. Samples 30-34 of Balady cultivar from Albassa site and samples 35-39 of Balady cultivar from Almarj site.

Results showed also that *E. necator* was the causal agent that produced different symptoms observed on grapevine including those on the Black cultivar of grapevine. At Kanawat 2 location, symptoms appeared on Black cultivar were pale to yellow patches on the upper side. On the underside, these areas were either covered or not covered with white to grayish fungal growth (Figure 4). In fact, this symptoms pattern is observed when the powdery mildew fungi have a tendency towards endoparasitism, such as in powdery mildew of Solanaceae family caused by *Leveillula taurica*. Accordingly, one can raise the question of whether the pathogen *E. necator* has a tendency for endoparasitism under certain environmental conditions. It is also observed that the symptoms produced may vary according to the grapevine training system used. Symptoms observed on Black cultivar with earth-trellised "Jui" training system in Kanawat 1 location were typical for powdery mildew, but they were atypical on the same cultivar in arbor vineyards at Kanawat 2 location.

**Figure 4**. Symptoms produced on Black cultivar of grapevine as pale to yellow patches on the upper side (right), and white to grayish fungal growth on the underside (left).

**Genetic Diversity of *E. necator* Isolates**

Twenty-nine isolates of *E. necator* were classified based on the observed variation in banding pattern with E07 primer and the constructed phenogram, using UPGMA. Isolates fell into four groups according to geographical locations and grapevine cultivar (Figure 5). Group A included seven Balady cultivar isolates collected from Kanawate 2 location, group B comprised of seventeen isolates, eight of Black cultivar from Kanawat 2 location; four of Black cultivar collected from RC/1 location; four of Balady Cultivar collected from RC/2 location; one isolate of Black cultivar collected from Kanawat 1 location. Group C comprised of four isolates of Black cultivar collected from RC/3 location. Group D comprised of one isolate from Balady cultivar in Kanawat1. Although this was a preliminary study because it was only based on using the primer E07, but the results were satisfactory and similar to other previous studies (Péros *et al*., 2005). Isolates from one cultivar and within one region had higher genetic similarity with each other than isolates from other areas. In group A, only isolates from Kanawate 2, in group C only isolates from Research Centre/3, in group D one isolate from Kanawat 1, but group B had more genetic diversity with isolates from all locations. Péros *et al*. (2005) based on RAPD results classified the *E. necator* isolates found in southern France into two groups (A and B). In Iran, Yousefi *et al*. (2010) showed that *E. necator* isolates were genetically distant in the surveyed geographical regions.

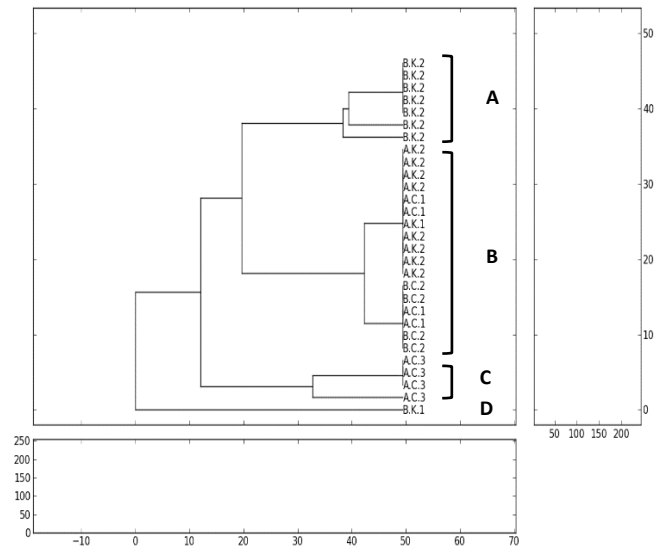


**Figure 5.** Phenogram generated by RAPD analysis of the 29 isolates of *Erysiphe necator*, using UPGMA method. BK2 = isolate of Balady cultivar collected from Kanawat 2 location, AK2= isolate of Black cultivar collected from Kanawat 2 location, AC1= isolate of Black cultivar collected from RC/1 location, AK1= isolate of Black cultivar collected from Kanawat 1 location, BC2= isolate of Balady cultivar collected from RC/2 location, AC3= isolate of Black cultivar collected from RC/3 location), and BK1= isolate of Balady cultivar collected from Kanawat 1 location.

The similarity observed between group B isolates from four geographical locations could be interpreted as a result of exchange and plantation of grapevine cuttings between the vineyards in these four areas. Consequently, some fungal isolates were moved and spread between those areas (Yousefi *et al.*, 2010). This hypothesis is supported by the findings of Delye *et al.* (1997), who mentioned that the conidia of *E. necator* are not easily disseminated by wind. Therefore, human interference such as trade of infected grapevine cuttings could cause its dispersal (Yousefi *et al*., 2010). This hypothesis may be supported by the fact that the

RC/3 location, not included in group B, is a small area for scientific research and not for grapevine plants production.

**Mating Type Detection of *E. necator* Isolates**

RAPD analysis of 39 *E. necator* isolates using the primer E07 showed the presence of 1000 bp band in only eight isolates (5, 6, 7, 8, 18, 19, 20, 21), and it was absent in all other isolates (Table 2).

**Table 2**. The presence (+) and absence (-) of the 1000 bp amplified fragment by primer E07.

| Isolate | Presence of 1000 bp band | Isolate | Presence of 1000 bp band |
|---|---|---|---|
| 1 | – | 21 | + |
| 2 | – | 22 | – |
| 3 | – | 23 | – |
| 4 | – | 24 | – |
| 5 | + | 25 | – |
| 6 | + | 26 | – |
| 7 | + | 27 | – |
| 8 | + | 28 | – |
| 9 | – | 29 | – |
| 10 | – | 30 | – |
| 11 | – | 31 | – |
| 12 | – | 32 | – |
| 13 | – | 33 | – |
| 14 | – | 34 | – |
| 15 | – | 35 | – |
| 16 | – | 36 | – |
| 17 | – | 37 | – |
| 18 | + | 38 | – |
| 19 | + | 39 | – |
| 20 | + | | |

All fungal isolates obtained from Black cultivar at the RC/1 location, and isolates from Balady cultivar at the RC/2 location were of mating type +, whereas the remaining isolates collected from other sites were of the mating type - (Figures 6 and 7). The + mating type are isolates that produced 1000 bp fragments when the primer E07 was used, and – mating type are the isolates that did not produce such fragment using the same primer. The frequency of each of these two mating types varied depending on the geographical location, with a predominance of the mating type - with frequency of 72.41% of presumably single spore isolates, and 79.49% of all studied isolates. The mating type + was found in 18.18% and 23.52% of isolates, and mating type - in 81.81% and 76.47% of isolates of Balady and Black cultivars, respectively.

Our results showed that all isolates in groups A, C and D were of mating -, but those in group B were either + or -. In an earlier study, two groups of *E. necator* isolates were found in southern France, all isolates in group A were of mating type +, but a high variation was observed in group B that included isolates of either mating type + or – (Péros *et al*., 2005). In Iran, 20 isolates were classified into seven groups. All isolates in groups A, D, F and G were of mating type -, but group B included the largest number of isolates of mating type (+) or (-). Results obtained in this study were similar to what has been reported earlier (Bouscaut and Corio-Costet, 2007; Cortesi *et al*., 2005). According to the results of Péros *et al.* (2005), most of our isolates belonged to group B, and they were able to reproduce sexually and/or asexually. Such results confirmed those of previous study showing that chasmothecia were formed on infected plant leaves in all studied locations, except Research Center/3, where no chasmothecia were observed (Alimad *et al.*, 2017).

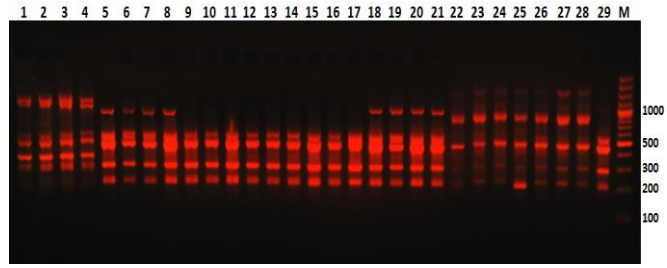


**Figure 6.** RAPD banding pattern generated by PCR using the primer E07. Lane M= molecular weight marker (1 kb-ladder); lanes 1-4= isolates from Research Centre 3 (Black cultivar), lanes 5–8= isolates from Research Center 1, lanes 9–16= isolates from Kanawat 2 (Black cultivar), lane 17= isolate from Kanawat 1 (Black cultivar), lanes 18–21= isolates from Research Centre 2 (Balady cultivar), lanes 22–28= isolates from Kanawat 2 (Balady cultivar), and lane 29= isolate from Kanawat 1 (Balady cultivar).

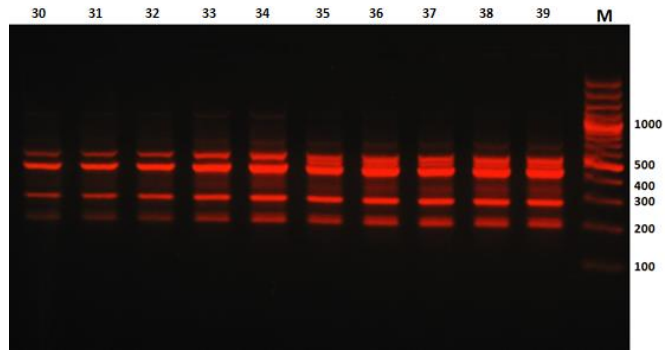


**Figure 7.** RAPD banding pattern generated by PCR using primer E07. Lane M= molecular weight markers (1 kb-ladder); lanes 30-34= isolates from Albassa (Balady cultivar), lanes 35-39= isolates from Almarge (Balady cultivar).

## الملخص

**العماد، نجود، وليد نفاع وسلام لاوند. 2021. الكشف عن الفطر *Erysiphe necator* المسبب لمرض البياض الدقيقي على كرمة العنب، وتحديد الأنماط التآلفية في جنوب سورية باستخدام بعض المؤشرات الجزيئية. مجلة وقاية النبات العربية، 39(2): 152−158.**

يُعد مرض البياض الدقيقي المتسبب عن الفطر *Erysiphe necator* أحد أهم أمراض كرمة العنب في كل أنحاء العالم. قورنت ثلاث طرائق لاستخلاص الـ DNA الفطري وهي: (1) كيت جاهز من شركة Promega ، (2) فينول كلوروفورم كحول الإيزوميل (PCI)، و (3) الـ SDS. بينت النتائج أن طريقة الـ SDS كانت الأفضل لاستخلاص الـ DNA الفطري، كماً ونوعاً، من أجل إجراء تفاعل البلمرة المتسلسل (PCR) والاختبارات الجزيئية الأخرى. أجري تفاعل البلمرة المتسلسل باستخدام زوج البادئات Uncin144 و Uncin 511، حيث تم الحصول على حزم بحجم 300−400 زوج قاعدي لـ 29 عزلة من الفطر *E. necator* متحصل عليها من مواقع جغرافية مختلفة، ومن أصناف مختلفة من الكرمة. أظهرت النتائج أيضاً أن الفطر *E. necator* يمكن أن يعطي أعراضاً غير نموذجية مشابهة لتلك المتسببة عن أمراض البياض الزغبي على بعض أصناف كرمة العنب تبعا للظروف البيئية ونظام التربية المتبع. توزعت عزلات الفطر *E. necator* في أربع مجموعات متباينة وراثياً تبعاً للصنف والموقع الجغرافي المدروس، وذلك بالاعتماد على تباين توضع الحزم في الهلام باستخدام البادئة E07، ورسم شجرة قرابة وراثية باستخدام طريقة UPGMA. أظهر تطبيق تقانة RAPD باستخدام البادئة E07 على 39 عزلة وجود نمطين تآلفيين (+ و −)، وقد تباينت النسبة المئوية لتردد كل منهما تبعاً للموقع الجغرافي، مع سيادة للنمط التآلفي السالب (-).



**كلمات مفتاحية:** بياض دقيقي، *Erysiphe necator* ، RAPD، نمط تآلفي، استخلاص الـ DNA.



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