Genetic diversity among *Spilocaea oleagina* isolates from different regions in Palestine

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Abstract

The peacock eye disease or the olive leaf spot caused by the fungus, Spilocae aoleagina, is the most olive destructive disease in many olive growing regions worldwide. The disease causes yield losses of up to 20%. Infection with the fungus depends on the environmental conditions and varies according to the different regions as well as olive genotype. The aim of this work was to study the genetic variations between the isolates from different olive growing regions in Palestine. In this work, six S. oleagina isolates were collected from different regions in Palestine including Qalqilia, Nablus, Tulkarm, Ramallah, Jenin and Salfit. Pure cultures of the fungus were obtained from single spore germination on PDA media. PCR was used to identify the fungal isolates using species specific primers. After using Box PCR, the diversity of the isolates was studied using the Box REPAIR primer. BLASTn search of S. oleagina isolates revealed similarities of 99% to S. oleagina (Accession #. AF338393.1). Based on the analyses of the dendrogram obtained after Box PCR, two distinct isolates were obvious. The first group (A) was divided into two subgroups (subgroup A1 included samples from Nablus and Ramallah and subgroup A2 included samples from Qalqilia, Salfit and Tulkarm). The second group (B) included a sample from Jenin only. To the best of the researchers' knowledge, this is the first work that identifies the genetic differences between S. oleagina isolates in Palestine. Further work is needed to examine the pathogenicity of the isolates on different olive genotypes.

Keywords: *Olive, peacock disease, Box PCR, single spore, genetic diversity*.

Introduction

The peacock eye disease or olive leaf spot (OLS), caused by the fungus *Spilocaea oleagina*, Castagne (Hughes) (syn. *Cycloconium oleagina*), is among the most common fungal diseases on olive trees [(*Olea europaea* L.) (Graniti 1993; Obanor *et al.*, 2008)]. Severely infected trees show defoliation, poor twig and growth (Figure 1). As a result of infection, yield losses may reach up to 20% (Azeri, 1993; Obanor *et al.*, 2008). Dark green or brown circular spots (2-15 mm in diameter) surrounded by a yellow halo on the upper surface of the leaves are the main symptoms of the disease (Salman *et al.*, 2011, Salman 2017). The disease may also affect the fruits in some cases, especially when the conditions are favorable for the fungus activity. Lesions produced by the fungus (Figure 1) are usually found on the upper surface of the leaf with muddy green to almost black circular spots (Graniti 1993). The infection with *S. oleagina* requires high humidity accompanied with temperatures in a range of 0-27°C. Specifically speaking, the temperatures from 16 to 21°C and 70-80% relative humidity are the optimal condition for OLS activity (Obanor *et al.*, 2008; Obanor *et al.*, 2011).

All olive growing regions in Palestine and most of olive cultivars are susceptible to the infection with *S. oleagina* (Abuamsha *et al.*, 2013; Salman *et al.*, 2011). Despite the spread of the fungus in different places in Palestine, there were variations in the severity of the disease in terms of the regions and resistance of olive trees to the fungus (Hajjeh *et al.*, 2014; Abuamsha *et al.*, 2013), which might be attributed to the variations in fungal strains based on its reproductive strategy and environmental conditions (Bernaschina *et al.*, 2019). Therefore, understanding the genetic diversity of the fungus is an important step before planning suitable control methods of the disease (McDonald and Linde 2002).

The analysis of DNA sequences of the rRNA genes (rDNA) as well as the internal transcribed spacer (ITS) regions are widely used to study Phylogenetic and evolutionary relationships of many organisms (Varga, 2000). It should be noted that the 18S and 28S rDNA sequences are of the most powerful indicators in taxonomy because they are universally found in living cells and are highly conserved (Bruns, 1991). Several DNA based markers have been used to evaluate genetic diversity of *S. oleagina*, since in vitro cultures do not sporulate (Dunkle and Levy, 2000; Arenal *et al.*,1999). In this study we report the classification of *S. oleagina* using the 16S rDNA sequences.

Figure 1: Symptoms of defoliation on an olive tree as a result of severe infection with OLS and leaves showing the typical lesions of the disease (source: the Author).

Materials and methods

Collection of the infected olive leaves and single spore culture

Freshly infected olive leaves with obvious symptoms of OLS were collected from six regions in Palestine during February-March 2018. To obtain spores from infected olive leaves, a drop (20 µl) of autoclaved distilled water was pipetted on leaves with obvious sporulating lesions and transferred to sterile glass slide. After that, a 1 µl loop was used to transfer spores to petri dish containing potato dextrose agar (PDA) media. The plates were sealed with parafilm and incubated at 20°C for 24 h in dark. The cultures were examined daily under light inverted microscope at 200X magnification. Contaminated cultures were excluded and the germinated spores were transferred to a new PDA plates by cutting the agar block that bears the germinated spores. The plates were further incubated at 20°C until the pure mycelia colony from each isolate was produced. The cultures were maintained on PDA and sub-cultured routinely every 2 months as mentioned by Obanor *et al*. (2010).

Confirmation of fungal species of *S. oleagina*

Mycelium of each fungal isolate was scrapped from the media and placed in a one ml Eppendrof tube containing 700 µl extraction buffer containing 20 M Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS and 10 µl proteinase K. The mycelia were ground using a pellet pestle homogenizer (Kimble). After that, 500 µl of phenol-chloroform was added to each tube and mixed by vortex for 5 min before centrifugation at 13000 rpm for 20 min. The upper phase was transferred to fresh tubes and 30 µl of 5 M sodium acetate (NaoAc) and 1 ml 70% ethanol were added. The tubes were centrifuged at 13000 rpm for 10 min and the pellets were washed with 70% ethanol. The DNA was dissolved in 50 µl TE buffer (pH 8, containing 10 mM Tris-HCl and 1.0 mM EDTA) and stored at -20°C until use (Sambrook *et al.*, 1989).

Molecular identification of the fungal isolates was done using the specific primers 18S F 5'-GCTTGTCTCAAAGATTAAGCC-3' forward and 18S R 5'-CCTTGTTACGACGACTT TTACTTCC-3' Reverse, that amplifies the 18S rDNA region (González-Lamothe *et al,* 2002; Obanor *et al.,* 2010). The PCR amplification was performed in a final volume 20 µl containing 10 µl GoTaq® Green (2X) Master Mix (Promega Corporation), 1 µl of each primer (10 pmol), 1 µl Fungal DNA (~50 ng) and 7 µl nuclease-free water. The amplification was carried out in thermal cycler (Veriti™ DxThermo Fisher Scientific) using the following program: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for30 sec, annealing at 55°C for 30 sec and primer extension at 68°C for 30 sec. A final extension cycle at 68°C was carried out for 7 min.

Genetic variation of *S. oleagina*

For the discrimination of fungal isolates, Box PCR using the Box REPAIR primer (5'-CTACGGCAAGGCGACGCTGACG-3') was used according to the method of Charan *et al*. (2011). PCR amplifications were carried out in 25 µl reactions. The reaction mixtures contained 3 µl fungal DNA, 1.5 µM of Box primer, 12.5 µl GoTag master mix and 8 µl nuclease-free water. Amplification conditions were as follows: initial denaturation at 94°C for 3 min, followed by 45 cycles of denaturation at 94°C for 25 sec, annealing at 52°C for 60 sec and primer extension at 72°C for 60 sec. A final extension at 72°C was carried out for 8 min. PCR products were separated and analyzed by TAE-agarose gel electrophoresis. Gel images were recorded for fingerprint profiles production. The images were recorded and imported into GEL Jv2 program.

Fingerprint profiles from BOX PCR results were created for isolated sample and the resulting profiles were analyzed using the (Pearson) similarity matrix followed by cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) to generate Phylogenetic tree (Bernaschina *et al*., 2019).

Results and Discussion

Single spore isolation and production of pure culture

Successful cultures were produced from single spores of OLS (Figure 2). Slow growing fungal cultures were obtained. The colonies were morphologically identical in all fungal isolates from different regions. A hemispherical grayish green of about 1.5 cm in diameter was produced (Figure 2). The mycelium was very compact and did not produce conidia under in vitro conditions. Within this context, the results are in agreement with the findings of Obanor *et al.* (2010).

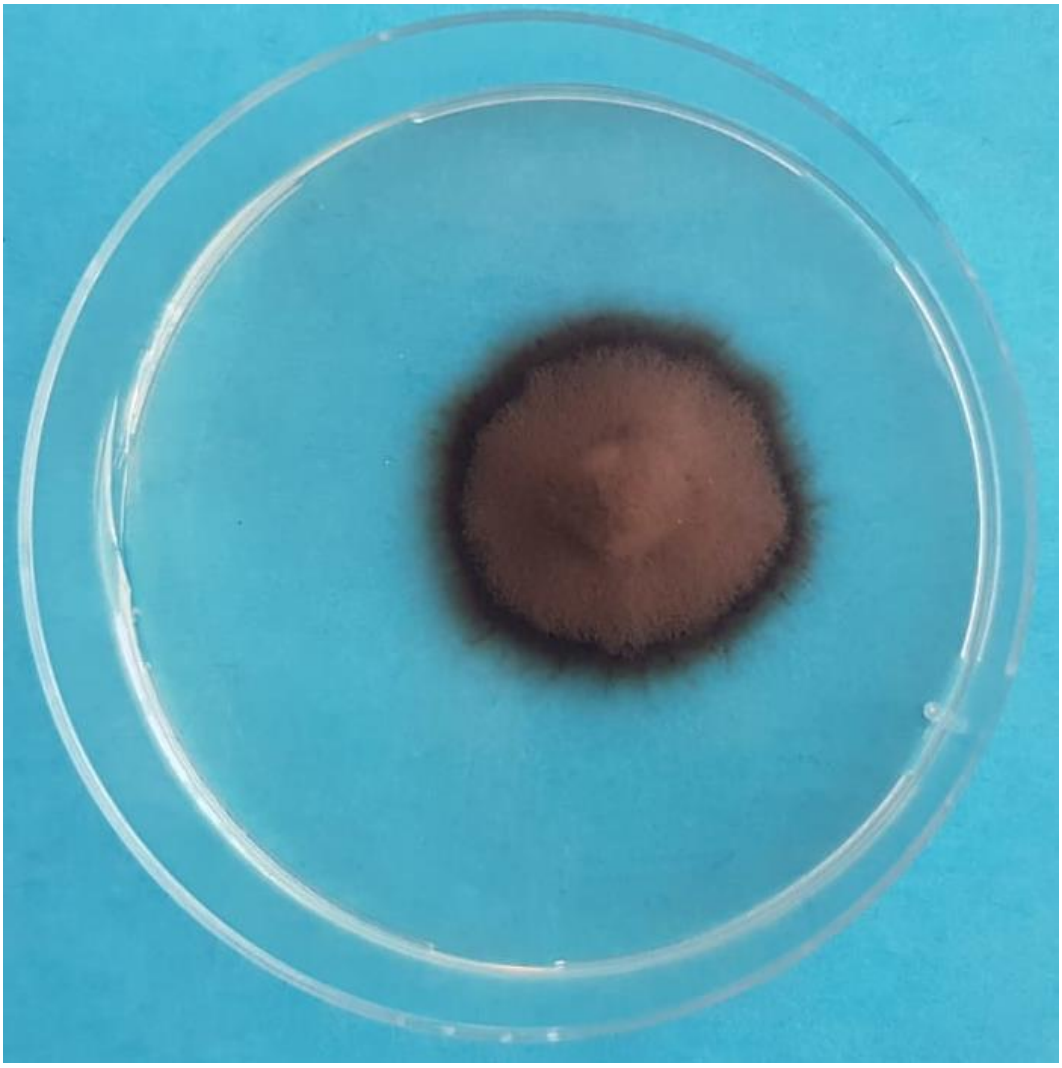

Figure 2: *S. oleagina* mycelia cultures on PDA media after 3 months of incubation at 20°C.

Fungal identification

In this study, a collection of six isolates of *S. oleagina* were obtained from olive leaves with typical symptoms of olive leaf spot. The fungal isolates were grown and maintained on PDA medium for further studies. Confirmation of the fungal species was done by PCR. The PCR products revealed bands of about 750 bp using the 18S F and 18S R specific primers (Figure 3). BLASTn search of *S. oleagina* isolates from Qalqilia, Nablus, Tulkarm, Ramallah, Jenin and Salfit revealed similarities of 99% to *S. oleagina* (Accession #. AF338393.1) (Figure 4).

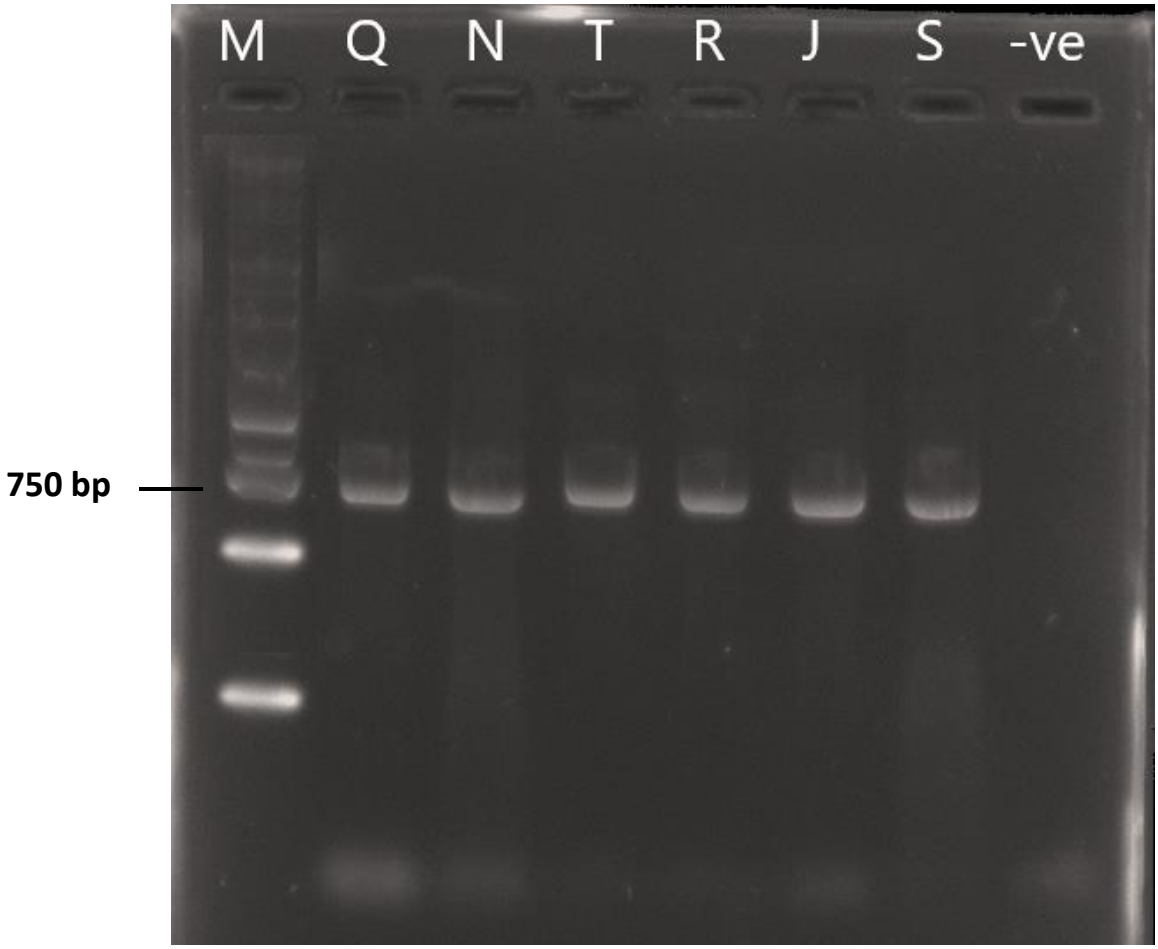

Figure 3: Gel electrophoresis of PCR products from different isolates of *S. oleagina* isolated from different regions in Palestine, Qalqilia Q, Nablus N, Tulkarm T, Ramallah R, Jenin J and Salfit S. M: 1kb marker.

Figure 4: BLASTn similarity of the sequence identity of *S. oleagina* isolated from Nablus.

Genetic diversity of the fungus

Discrimination between different isolates of *S. oleagina* was done using Box PCR. The banding pattern (Figure 5) revealed differences after gel electrophoresis of the Box PCR product.

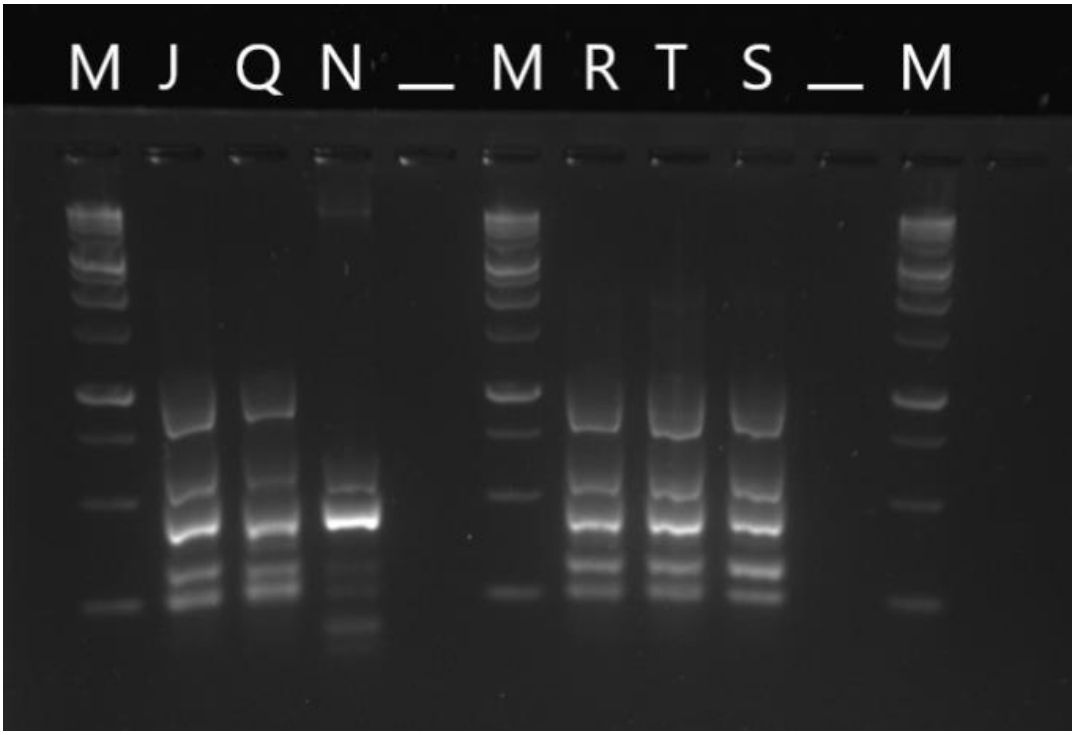

Figure 5: Separation of Box PCR products of six *S. oleagina* isolates. (M: 1Kb marker. Lanes J, Q, N, R, T, and S are Jenin, Qalqilia, Nablus, Ramallah, Tulkarm, and Salfit, respectively).

BOX-PCR amplification of *S. oleagina* isolates generated some distinct fingerprint patterns. The cluster analysis divided the fingerprints of the isolates into two groups (Figure 6): group A, which included two subgroups (A1 and A2), and group B. The subgroup A2 contained 3 isolates and was the largest cluster. The remaining groups, in decreasing number of isolates, were subgroup A1 (2 isolates) and group B (1 isolate). All of the three groups contained fungi isolated from host genotypes originally from different governorates in Palestine. That is, in group one, *S. oleagina* samples were isolated from the olive trees originating from Ramallah and Nablus, however in group two, the samples were isolated from Salfit, Tulkarm and Qalqilia, and finally in subgroup A2, the samples were isolated from Jenin.

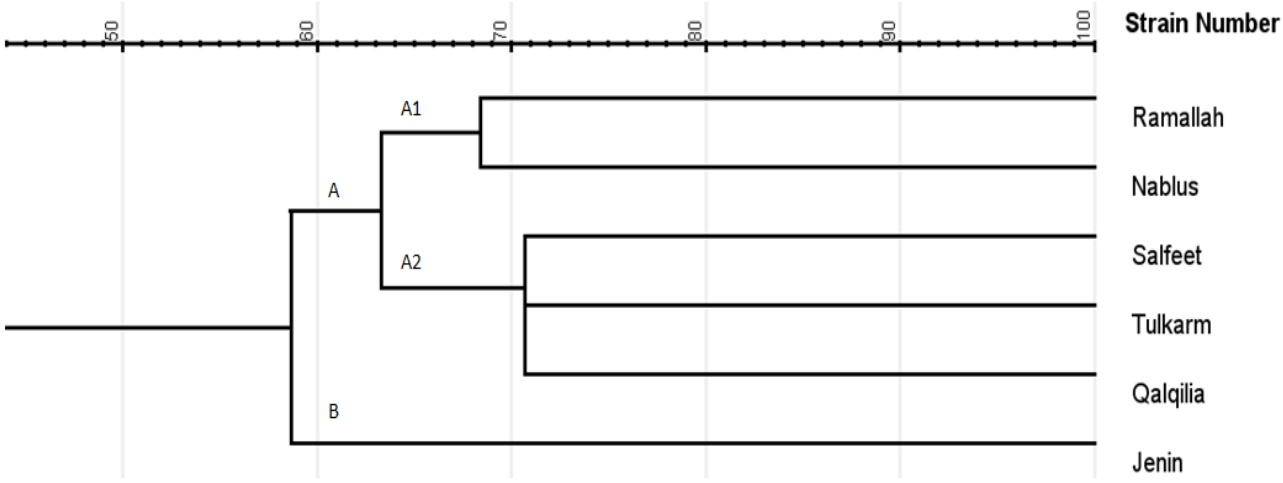

Figure 6: Phylogenetic tree indicating the relatedness among *S. oleagina* isolates based on Box-PCR analysis.

The olive leaf spot is wide spread in Palestine (Salman *et al.,* 2011; Abuamsha *et al.,* 2013; Hajjeh *et al.,* 2014). Five regions (Qalqilia, Tulkarm, Nablus, Jenin and Salfit) were considered as highly affected by the disease (Salman *et al.,* 2011). The severity and index of the disease in these regions were higher than in other regions in Palestine (Salman *et al.,* 2011) due to the weather conditions (high humidity and cool) in these regions, which favor the development of the disease over other places in Palestine (Hajjeh *et al.,* 2013).

The nucleotide sequences of the 16S region of rDNA were obtained from six *S. oleagina* isolates except for the fungal isolate from Qalqilia. Consequently, the comparative analysis of these sequences showed a high degree of similarity. Box PCR has also proved to be a rapid and highly reproducible molecular biologic technique for the discrimination of the genotypic variation among the different species. Its capacity to discriminate between similar, but not identical, isolates meet or even exceeds that of the other available molecular typing approaches (Koeuth *et al*., 1995). In addition to that, the differences in fungal genotypes could be due to the selection of pathogen genotypes by the environmental conditions and disease management in the different regions. In other words, each group or subgroup of the isolated fungus consisted of regions that are similar in climatic conditions. For example, the subgroup A1 (Nablus and Ramallah) was more closely related when compared with the subgroup A2 (Tulkarm, Qalqilia and Salfit). The low genetic diversity might result from asexual mode of reproduction that predominates among *S. oleagina* (Graniti, 1993). Another explanation of the variation in pathogen populations might be generated by mutation, migration and recombination (sexual or asexual) (Obanor *et al.,* 2011). Based on the researchers' results, it is possible that these forces have not acted sufficiently to produce high variations among the same group of the Palestinian isolates. However, they might act to produce variations between the different groups of the isolates.

Conclusion

Our study is the first report in Palestine on genetic diversity of *S. oleagina*. This study has showed that *S. oleagina* populations had a low level of genetic diversity among isolates, probably because of asexual reproduction, which is the major method by which this fungus spreads its populations. The low genetic diversity in *S. oleagina* populations suggests that any effective control strategies are likely to be useful in all or most olive groves. However, further

studies are needed to evaluate prober control plans in different regions of Palestine according to the severity and occurrence of the disease.

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التنوع الوراثي بين عزلات فطر *Spilocaea oleagina* من مناطق مختلفة في فلسطين

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الملخص

يعد مرض عين الطاووس الذي يسببه فطر Spilocaea oleagina من أكثر الأمراض الفطرية التي تصيب الزيتون أهمية في جميع المناطق التي يزرع فيها الزيتون. وتصل نسبة الخسائر في المحصول إلى ما يزيد عن 20%. تعتمد الإصابة بالمرض على الظروف المناخية وتختلف باختلاف المنطقة وكذلك صنف الزيتون. وهدفت هذه الدراسة إلى تحديد الاختلاف الوراثي بين عزلات من الفطر في مناطق زراعة الزيتون المختلفة في فلسطين. تم في هذا البحث جمع ست عزلات من الفطر من مناطق مختلفة في فلسطين شملت قلقيلية، ونابلس، وطولكرم، ورام الله، وجنين، وسلفيت. وتم الحصول على عزلات نقية من الفطر عن طريق إنبات أبواغ الفطر على بيئة PDA. وقد تم استخدام ال PCR لتوصيف هذه العزلات. وتم بعد ذلك دراسة الاختلاف الجيني بين العزلات باستخدام Box PCR. وقد أظهرت نتائج التوصيف وجود تشابه بنسبة 99% مع فطر Spilocaea oleagina حسب موقع بنك الجينات (BLASTn). وبعد تحليل نتائج الاختلاف الجيني بين عزلات الفطر تبين أن هذه العزلات يمكن تقسيمها إلى مجموعتين. المجموعة الأولى A وتنقسم إلى مجموعتين (A1 وتضم العزلات من محافظة نابلس ورام الله و A2 تضم العزلات من قلقيلية، وسلفيت وطولكرم). أما المجموعة الثانية B، فتضم عزلة الفطر من محافظة جنين. وحسب معلومات المؤلفين فإن هذه الدراسة هي الأولى من نوعها في فلسطين، التي تشخص مرض عين الطاووس في فلسطين من ناحية جينية وتبين الاختلاف بين عزلات الفطر من مناطق مختلفة، وذلك تمهيدا لإيجاد وسائل مناسبة لمكافحة المرض بالطرق الحديثة.

الكلمات المفتاحية: *الزيتون، مرض عين الطاووس، Box PCR، بوغ منفرد، تنوع وراثي.*