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Cross-transferability of SSR loci of *Phaeosphaeria nodorum* to *Mauginiella scaettae*

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Abstract: *Mauginiella scaettae* is one of the most critical and devastating fungal pathogens causing date palms inflorescence rot (khamedj). This pathogen, in severe attacks, can cause 80% loss of the annual harvest. In this study, seven SSR loci (have previously been isolated and characterized in *Phaeosphaeria nodorum*) were evaluated for transferability on 13 single-spore isolates of *M. scaettae* obtained from eight different regions of Khuzestan province, Iran. A high level of transferability of SSRs was detected. Five primer pairs, including SNOD1, SNOD26, SNOD22, SNOD17, and SNOD21, were successfully amplified and produced an amplification product of the expected size range in thirteen isolates collected from eight locations. Two microsatellite markers, including SNOD5 and SNOD16, were not amplified and showed no amplification. The rate of amplification of five amplified SSR loci was different among isolates. A total of sixteen alleles were obtained across the five SSRs loci for thirteen isolates. Among all isolates examined, the highest rate (92.3%) and the lowest rate (7.7%) of amplification were done for SNOD26 and SNOD21 SSR loci, respectively. The loci SNOD1, SNOD26, and SNOD22 generated four, and SNOD17 locus generated three alleles, and the lowest number of alleles (one allele) was identified in the SNOD21 locus.

Keywords: Date palm, Khamedj disease, SSR locus, ITS-rDNA, allele diversity

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) provides an excellent source of food with high nutritional values, called a mine (Zaid & Wet 2002). In addition to providing a wide range of essential nutrients, various parts of the date plant can be used in different industries. To eradicate food insecurity, malnutrition, and food crises, FAO (The Food and Agriculture Organization) has a unique look for the date palm and implement different projects and programs in various fields (Arias et al. 2016). Khuzestan province, located in the southwest of Iran, is considered as one of the most critical palm cultivation areas in Iran, which its date fruits are exported to different countries. Khuzestan province seems to be one of the places of origin and distribution of date in the world (Zaid & Wet 2002). The significant date palm cultivation areas in Khuzestan include the southern, western, and central parts of the province. One of the most important diseases of palm in Khuzestan province is khamedj disease (inflorescence rot). Based on our field observations, due to the favorable environmental conditions for the pathogen, Khuzestan province has a higher incidence of disease than other Iran provinces.

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Climate change and heavy rainfall during the last two years in this province led to a conducive environment and pathogen outbreak. Khamedj is the most severe disease in the hot and humid regions, and its outbreaks could occur after a prolonged cold and humid winter (Djebri 1998), the same condition that exists in Khuzestan. Severe outbreaks of the disease occurred in Basrah, Iraq, in 1948–1949 and 1977–1987, causing 80% loss of the annual harvest (Al-Hassan & Waleed 1977). The significant causal fungus of khamedj disease is *Mauginiella scaettae* (Ascomycota) (Abdollah et al. 2005).

Simple sequence repeats (SSRs) or microsatellites are short motifs (1–6 base pairs) repeated DNA sequences which their length variation occurs primarily due to slipped-strand mispairing during replication, repair, and recombinant genetic mutations (Rossetto 2001, Karaoglu et al. 2005). It has been demonstrated that SSR markers can be a valuable and efficient source for genetic characterization of species and other aspects such as detecting genetic variation in a pathogen population (Adhikari et al. 2008, Kumar et al. 2013). Karaoglu et al. (2005) have shown that the abundance of SSRs in fungi is low in comparison with other organism's genomes. The cross-transferability of SSRs have been widely recognized. It has been proven that SSR primers developed for one species can be used to study polymorphism at homologous loci in related species (Rossetto 2001). Thus, using such SSRs primers that have been previously developed in related species has become fast, efficient, and cheaper. Hence, because of the lack of sufficient information on the *M. scaettae* genome, seven SSR primers previously reported in *Phaeosphaeria nodorum* (Stukenbrock et al. 2005, Adhikari et al. 2008) were evaluated for the cross-transferability to *M. scaettae*. Microsatellites present in expressed regions of the genome (Expressed Sequence Tags or EST-SSR) have a crucial advantage compared with SSRs from non-coding sequences which is that they are often more transferable across species (Kumar et al. 2013, Singh et al. 2014). EST-SSRs have been used in various genetic studies in different organisms, and a high rate of their transferability has already been reported for animals, plants, and fungi (Cristancho & Escobar 2008).

The present study aimed to evaluate EST-SSRs transfer between *P. nodorum* and *M. scaettae* isolates and used this powerful marker to investigate the genetic diversity of *M. scaettae* isolates collected from eight regions of the most critical locations of date palm cultivation in Khuzestan province in Iran.

MATERIALS AND METHODS

Sampling and isolation of *Mauginiella scaettae*

Sample collection was made in eight regions of Khuzestan province in the southwest of Iran from March to May during 2018-2019 (Fig. 1; Table 1). Samples were collected from date palms showing symptoms of blackish rust spots on the spathe's outer

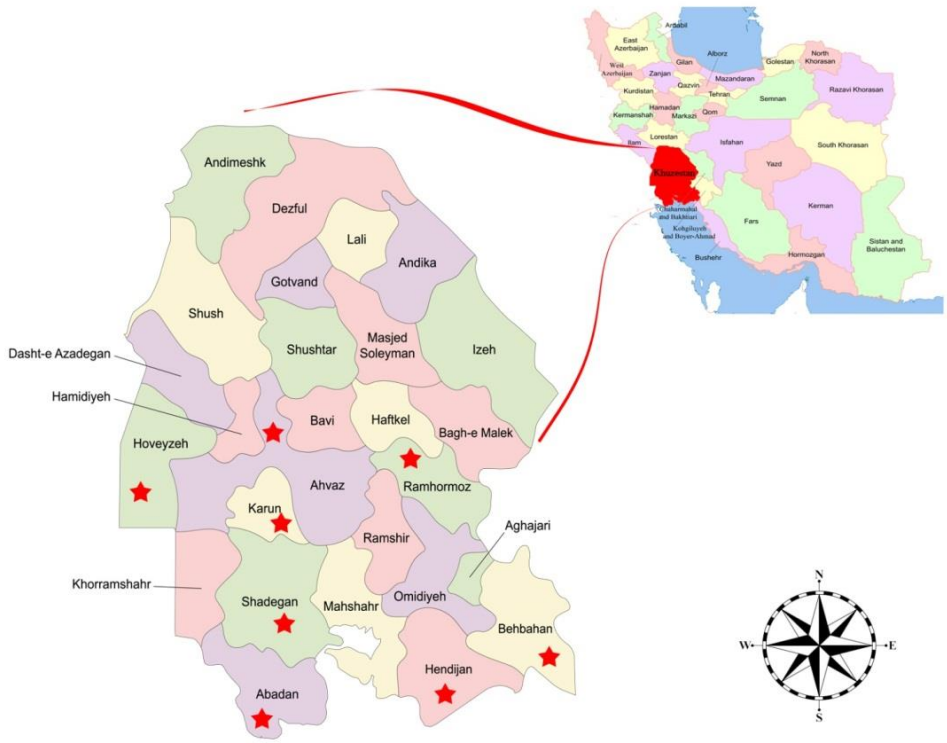

Fig. 1. Sampling locations of *Mauginiella scaettae* in palm date cultivation regions of Khuzestan province. The sampling locations have shown by red stars (https://en.wikipedia.org).

Table 1. Characterization of the isolates of *Mauginiella scaettae* used in the present study. All isolates were obtained in different regions of Khuzestan province, Iran.

Sampling region	Isolate	Palm variety	Geographic position	Sampling time
Abadan	abad-sa1	Sayer	30.0792-48.2518	2018
Abadan	abad-ma1	Male	30.2504-48.2621	2018
Ahvaz	ahva-ma1	Male	31.3100-48.6596	2019
Ahvaz	ahva-med1	Medjool	31.2508-48.5473	2019
Behbahan	behb-kh1	Khasi	30.7451-50.1646	2018
Behbahan	behb-kh2	Khasi	30.7450-50.1675	2018
Behbahan	behb-ma1	Male	30.7479-50.1634	2018
Hoveyzeh	hove-gh	gheibani	31.4689-48.0746	2019
Karun	karo-sa1	Sayer	31.2278-48.6404	2018
Karun	karo-khad1	Khadrawi	31.2278-48.6404	2018
Hendijan	hend-br1	Bream	30.1434-49.4341	2019
Ramhormoz	ramh-sa1	Sayer	31.2749-49.6035	2018
Shadegan	shad-sa1	Sayer	30.61325-48.6624	2018

surface and slightly deformation to rotten on the inflorescences (spathes). The main symptoms were rotten spathe which covered by abundant white powder produced by the arthrospores of *M. scaettae* (Fig 2). To isolate the fungus, the infected tissues from each spathe were cut into small pieces (7-8 mm) and surface-disinfested in 2% sodium hypochlorite solution for 3 min, rinsed twice with sterile distilled water, and dried in the side of two sterile filter papers. Five pieces were placed on potato dextrose agar (PDA, Merck Co.), amended with streptomycin sulfate (0.4 mg/L) and incubated at 25°C. After 5-7 days, newly grown mycelium and arthrospores were transferred and placed on new PDA media to obtain monoconidial isolates. The plates containing monoconidial isolates were preserved at 25°C. Additionally, to isolate the fungus, in cases where the arthrospores masses formed on the rotted spathes, the mass was transferred on PDA media without any surface-disinfested. All isolates were maintained on PDA slants at 4°C.

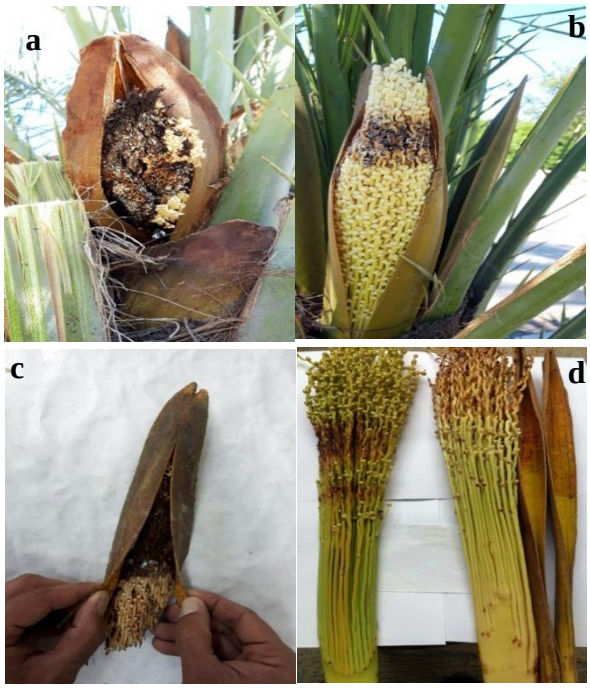

Fig. 2. Symptoms and signs of the khamedj disease of date palm. a. opened and unopened rotten male inflorescence which deformed slightly and showing blackish rust spots on the outer surface and covered by white *Mauginiella scaettae* arthrospores, b. Rotten female inflorescence, c. Opened rotten female inflorescence that severely destructed, d. Symptoms and signs on the different part of the inflorescence (spathe, spikes (flowers and strands).

DNA extraction

The mycelium was scratched from the surface of a 20-day-old PDA plate and powdered in liquid nitrogen. The DNA was extracted using phenol-chloroform protocol, according to Zhong & Stephenson (2001). The quality of DNA was determined using 2% agarose gel, and its quantity was adjusted to 50 ng by NanoDrop.

Morphological and molecular characterization

The identification of the *M. scaettae* was made based on the morphological criteria, colony morphology, mycelium, and arthroconidia according to Abdollah et al. (2005), Sigler & Carmichael (1976), and von Arx et al. (1982).

Internal transcribed spacer ITS-nrDNA region of four isolates, including abad-sa1, ahva-ma1, behb-ma1, and hove-gh, was sequenced to confirm the morphological identification. The ITS1-5.8S-ITS2 region was amplified using the ITS1 (5′-CTTGGTC ATTTAGAGGAAGTAA) and ITS4 (5′-TCCTCCGCT TATTGATATGC) primer pair (White et al. 1990) for 8 min at 95°C for the initial denaturation step followed by 35 cycles of 95°C for 30 s, 58°C for 20 s, 72°C for 60 s and a final extension of 72°C for 5 min.

SSR analysis

Seven primer pairs used in the current study are listed in Table 2. All EST-derived microsatellite loci which analyzed in this study were previously isolated and characterized from the wheat pathogen fungus, *Phaeosphaeria nodorum* (Stukenbrock et al., 2005). PCR reaction was carried out in a total volume of 20 µL containing 3 µl genomic DNA (50 ng), 0.5 ml of each primer (10 µM primers), 6 µL water, and 10 µL

Table 2. Characterization of seven microsatellite loci of *Phaeosphaeria nodorum* which their transferability to *Mauginiella scaettae* was investigated.

Locus	Primer Seq (5' - 3')	Annealing Temp (°C)	No. of alleles	size (bp)	Polymorphism (%)	Motif	PIC[a]
SNOD1 DR046161	F: CCATCATGTTGTCACGCTTAGC R: CGGTTACGGCAACAACAAGCC	58	4	252-342	30.77	TGT	0.94
SNOD26 DR045700	F: CGTCATCGGCACCACCAGCCAT R: CATTGTGCATCGTGCATCA	62	4	208-242	30.77	CAC	0.78
SNOD5 DR045205	F: GGACCTTGAGTAAAGGCTGGC R: GAATGGACAATTTCCAGCTATGC	-	-			GTC	
SNOD16 DR045916	F: TCTGTCGTCTGTGTCATTCTG R: GTAAACGGATCCTCCCACC	-	-			CT	
SNOD17 DR045078	F: CAACGGCCAGAAT R: GTGGCAACAACAACGCT	60	3	92-182	23.08	GTT	0.94
SNOD22 DR045164	F: GCACTTTCGAGAACACCTTC R: CAAATGGATAGTCTGCGCA	52	4	231-255	30.77	CAC	0.97
SNOD21 DR074925	F: CCGCAAACATACAAATCATC R: CACATCCCATTACACAAT	44	1	191-218	7.69	ACC	0.99

[a] PIC: Polymorphic information content

master mix (1.5X). PCR reaction was programmed as initial denaturation for 2.5 min at 96°C followed by 36 cycles of denaturation for 30 s at 96°C; annealing for 1 min (modifications of annealing temperatures were necessary and different depending on used markers (Table 2)), extension for 1 min at 72°C followed by 5 min at 72°C. The amplified products were separated on 4% polyacrylamide gel. The gels were run in 1X TBE buffer in electrophoresis apparatus at 250 V for two h and 30 min, stained with silver nitrate (Hung et al. 2018).

Statistical analysis

All band lines were visually scored as present (1) or absent (0). Data of SSR markers were entered into Microsoft Excel version 2010. The data were analyzed using NTSYS-pc software version 2.2.

The average of alleles per locus and the polymorphic information content (PIC) were estimated and compared between EST-SSRs. The data were analyzed using a qualitative data route to generate Jaccard's similarity coefficient using NTSYS-pc, software version 2.2. The corresponding dendrograms were constructed by the unweighted paired group method with the arithmetic mean (UPGMA) and SAHN clustering.

RESULTS

Morphological and molecular identification

Thirteen isolates of *M. scaettae* were recovered from seven cultivars at eight locations of Khuzestan province during 2018-2019 (Table 1.). The mono-conidial isolates were identified and verified based on morphology (Abdollah et al. (2005), Sigler & Carmichael (1976), and von Arx et al. (1982)). The fungus colonies on PDA were relatively slow-growing, reaching 9 cm in diameter after ten days at 25°C, white; the reverse, creamy, and in some isolates becoming brown to black in old cultures PDA. The hyphae were regularly septate and repeatedly branched. Many abundant arthrospores were produced by segmentation of the hyphae. The arthrospores were unicellular or multicellular (1-6 cellular and rarely up to 13 cellular), hyaline, cylindrical, or ellipsoidal and rarely round. The identification was confirmed by sequencing the ITS-nrDNA region. The size of the amplified region was found to be varying from 564 to 606 bp in the four monosporic isolates. The sequence data alongside with BLAST search showed more than 99 % homology with *M. scaettae* and proved the identity of *M. scaettae* isolates which confirmed the morphological identifycation.

Transferability of SSR loci

Seven primer pairs (Table 2) were used on thirteen isolates of *M. scaettae* (Table 1), and *Trichoderma* sp. were tested as a control isolate. Five of the primer sets (˜71.4%) successfully amplified and generated DNA bands with expected size range and two EST-SSR markers (˜28.57) showed no amplification (Table 2). The lowest number of alleles (one allele) was detected at the SNOD21 locus, and the loci SNOD1, SNOD26, and SNOD22 generated more allele numbers (four alleles). The three alleles were detected at SNOD17 locus. The highest amplification percentage was in SNOD26 primer, which showed amplification for 92.3% of the tested isolates (Fig. 3). The low amplification rate was in SNOD21 primer, which generated the expected band in only one isolate (7.7% of the tested isolates). The EST- SSR markers, PCR annealing temperature, fragment length, percentage of polymorphism, and PIC are described in Table 2. Eventually, the five EST- SSR loci revealed a total of sixteen alleles with an average of 3.2 alleles per locus.

Fig. 3. SSR banding patterns detected by SNOD26 microsatellite in *Mauginiella scattae* isolates. Lane 1: hov-gh; Lane 4: ahva-med1; Lane 8: abad-ma1; Lane 12: behb-ma1; Lane 13: ramh-sa1; Lane 14: behb-kh2; Lane 17: behb-kh1; Lane 19: ahva-ma1; Lane 20: shad-sa1; Lane 21: karo-sa1; Lane 23: hend-br1; Lane 26: abad-sa1; Lane 29: karo-khad1. M: DNA size marker.

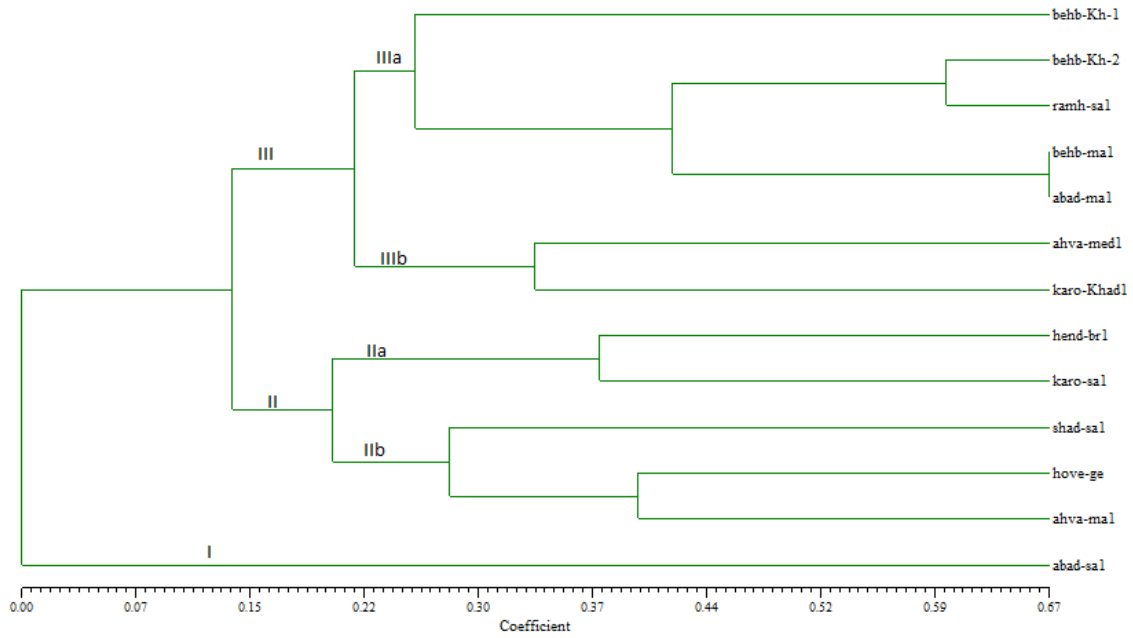

Fig. 4. Dendrogram showing a genetic relationship among thirteen *Mauginiella scaettae* isolates based on five microsatellite markers. Scale indicates Jaccard's coefficient of similarity. Group I, II and III represent three groups of isolates clustered.

Cluster analysis

The five EST-SSR markers, which successfully amplified, were used to assess the genetic diversity in thirteen *M. scaettae* isolates collected from eight locations (Table 1). The similarity coefficient values between isolates ranged from 0 to 0.66, with a mean of 0.38 for all thirteen isolates/SSRs. The highest similarity value was observed between isolates behb-ma1 and abad-ma1 (0.66), followed by isolates abad-ma1 and ramh-sa1 (0.57). The dendrogram (Fig. 4) constructed based on the similarity index resulted in three major clusters. The first cluster was separated from the other two groups and consisted of a single isolate abad-sa1. Cluster II included five isolates, and it was divided into two sub-clusters IIa, and IIb, at a cut-off similarity index of 0.06. Sub-cluster IIa comprised two hend-br1 and Karo-sa1; sub-cluster IIb comprised three isolates viz. shad-sa1, hove-gh, and ahvaz-mal from different regions of Khuzestan province. Cluster III contained seven isolates which was divided into two sub-clusters, IIIa and IIIb, at a cut-off similarity index of 0.20. Sub-cluster Ia comprised five isolates, including behd-kh-1, behb-kh-2, ramh-sa1, behb-mal, and abad-mal. Sub-cluster Ib consisted of two isolates from ahva-medl and karo-khad1 from Ahvaz and Karun cities, respectively.

DISCUSSION

The khamedj sampling time in Khuzestan is limited from February to April, and in the female date palms, it is difficult to diagnose the disease, if the fruits pass the hababauk stage and form the kimri stage. Hababauk is the term used for the female inflorescence, and it is the first stage of the fruit formation, and its development stages are after pollination. Kimri is one of the stages of maturation that its formation is after the hababauk stage, and at the end of the kimri, the date seed could already germinate and the fruit is botanically mature (Zaid & Wet 2002). Moreover, the increase of the temperature in summer causes inhibition of fungi germination, and it can be one of the disease limitations factors.

Several advantages of SSR markers, such as multiallelic, highly polymorphic, co-dominant, and highly reproducible, make them ideal molecular tools for different purposes. Additionally, the cross-transferability of SSRs has been widely recognized. Several studies report informative transferability of SSRs to very animal taxa (Peakall et al. 1998).

Peakall et al. (1998) also revealed that the Cross-species amplification between plant species is ranged from 50% to 100%. On the other hand, Dutech et al. (2007) study has shown that SSR cross-species transfer within fungal genera is low (34%). Despite the development and characterization of new SSRs loci across an entire genome are efficient, it is relatively expensive and will take a long time, especially in fungi, which SSR appeared harder to isolate (Dutech et al. 2007). Using SSRs that have previously been developed in closely related species seems to be faster, cheaper and more efficient, and mainly, it has been proven that SSR primers developed for one species can be used in related species (Rossetto 2001). Furthermore, researchers have suggested that the possibility of the cross genus and cross-species transferability of SSRs from closely related non-source species becomes advantageous (Baird et al. 2010). Hence, because of the lack of sufficient information on *M. scaettae* genome, in the current study, seven SSR primers reported previously

in *Phaeosphaeria nodorum* (Stukenbrock et al. 2005, Adhikari et al. 2008) were evaluated for their cross-transferability to *M. scaettae*. The use of *Phaeosphaeria* EST-SSRs to *M. scaettae* was as a result of researcher's belief that although the teleomorph stage of *M. scaettae* is unknown, *M. scaettae* represents an anamorph of a *Phaeosphaeria* species, and these two genera are closely related (Abdollah et al. 2005). Five SSR loci, including SNOD1, SNOD26, SNOD17, SNOD22, and SNOD21 were successfully amplified and revealed a high ability to transfer among these two genera. As mentioned above, it has been shown that the cross-species transfer of SSRs in fungi is low (Dutech et al. 2007). On the other hand, EST-SSRs have a high ability to transfer between species as compared with SSRs. The researchers considered that EST-SSRs are more efficient than genomic SSRs in terms of transferability among related species. It seems; this is due to the conserved nature of gene-associated sequences (Dracatos et al. 2006, Wang et al. 2010). For example, Kumar et al. (2013) shown that the transferability of *Fusarium oxysporum* EST-SSR to *F. udum* ranged from 60% (Foc primer) to 70% (Fol primer). Similarly, Dracatos et al. (2006) proved an average transfer frequency of 85% across the tested EST-SSR primer sets, and other investigations proved similar results (Kumar et al. 2013, Singh et al. 2014). One possible explanation for high transferability in current research could be that the two tested fungi (*P. nodorum* and *M. scattae*) are evolutionarily related because it has been proven that the success of amplification depends upon the evolutionary distance between the source and the target species (Rossetto 2001). Furthermore, since SSRs have a high rate of transferability between closely related fungi, they can give us informative knowledge about gene flow and evolution (Baird et al. 2010, Dutech et al. 2007) and consequently, they can consider as powerful tools for inferring about the represent anamorphic to unknown teleomorph. Our finding was also consistent with the study of cross–transferability of *Bipolaris sorokiniana* to related species, which were tested in Fajolu et al. (2013) study. Consequently, our finding confirms this theory that EST-SSR are highly transferable to other related species.

The teleomorph stage of *M. scaettae* is unknown. However, the cell wall's ultrastructure and the hyphal septa, together with the diazonium blue B test, have shown that *M. scaettae* represents an anamorphic of an unknown Ascomycete (von Arx et al. 1982). Furthermore, analysis of the internal transcribed spacer (ITS) region data indicates that *M. scaettae* is closely related to species of *Phaeosphaeria* (Abdollah et al. 2005). An exciting aspect of the present study is the confirmation of the close relation among *Mauginiella* and *Phaeosphaeria*, for the reason that the more closely related the organisms, the higher the rate of the transferability; because more closely related species sharing more homology in microsatellite loci (Singh et al. 2014).

The researchers proved that in 65% of the studies evaluating cross-species transferability of SSRs, modification of PCR protocols was necessary (Rossetto 2001, Baird et al. 2010). We could amplify all SSR loci by increasing the annealing temperature (SNOD1 (+2°C), SNOD26 (+6°C) and SNOD17 (+4°C)) of the PCR, and amplification of another two loci by lowering the annealing temperature (SNOD22 (-4°C), and SNOD21 (-12°C). Consequently, these five primer pairs, which demonstrated successful cross-species amplification, were used to further genetic diversity examine in different geographical locations and cultivars. Herein demonstrated that though results using EST-SSR confirmed the high genetic diversity of *M. scaettae* isolates, this genetic diversity was not strongly correlated to geographic origin, cultivars, or age of fungal isolation. Regarding the results of the similarity coefficient between *M. scaettae* isolates, all of the thirteen isolates used in the current study had a high level of genetic diversity. Furthermore, the constructed dendrogram (Fig. 4) indicated that all tested isolates were separated into three different significant clusters with the genetic distances of 0.136-0.33 among them. Therefore, in this study, we obtained a robust, reliable, and inexpensive tool for studying genetic diversity in this fungus by identifying and introducing these microsatellite loci.

To our knowledge, no molecular marker is available to study the *M. scaettae* fungi, and the present study was the first to evaluate molecular marker in *M. scaettae* and comparing the isolates of this pathogen based on geographical locations and date palm cultivars. In conclusion, EST-SSRs provided an essential tool for understanding the evolutionary contribution and investigating gene flow within the related species and can be used for additional studies to the resolution of genetic relationships. Understanding of genetic entity and genetics studies of *Mauginiella* are useful for effective khamedj management and developing control strategies for this disease causal agent. All of those understandings lead to more quick responses to future khamedj outbreaks.

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قابلیت انتقال‌پذیری جایگاه‌های SSR از *Phaeosphaeria nodorum* به *Mauginiella scaettae*

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چکیده: قارچ *Mauginiella scaettae* یکی از مهم‌ترین و مخرب‌ترین عوامل بیماریزای قارچی است که باعث پوسیدگی گل‌آذین نخل خرما می‌شود. این عامل بیماری‌زا در شرایط آب و هوایی مساعد می‌تواند باعث از بین رفتن ۸۰ درصد برداشت سالانه محصول خرما شود. در این پژوهش، هفت جایگاه EST-SSR که قبلا در قارچ *Phaeosphaeria nodorum* شناسایی شده بودند، از نظر قابلیت انتقال به سیزده جدایه *M. scaettae* به دست آمده از هشت منطقه استان خوزستان بررسی شدند. سطح بالایی از انتقال در EST-SSRها مشاهده شد؛ به طوری‌که پنج آغازگر SNOD1، SNOD26، SNOD22، SNOD17 و SNOD21 با موفقیت تکثیر شده و باندهای مورد انتظار مربوط به خود را ایجاد کردند. دو نشانگر ریزماهواره SNOD5 و SNOD16 نیز تکثیر نشدند. میزان تکثیر پنج نشانگر EST-SSR در بین جدایه‌ها نیز متفاوت بود. در مجموع شانزده آلل از پنج لوکوس EST-SSR برای سیزده جدایه به دست آمد. بیشترین میزان (۹۲/۳ درصد) و کمترین میزان (۷/۷ درصد) تکثیر جایگاه SSR در جدایه‌ها، به ترتیب مربوط به آغازگرهای، SNOD26 و SNOD21 بود. همچنین جایگاه‌های SNOD1، SNOD26 و SNOD22 چهار و SNOD17 سه آلل ایجاد کردند و کمترین تعداد آلل (یک آلل) مربوط به جایگاه SNOD21 بود.

کلمات کلیدی: خرما، بیماری خامج، جایگاه SSR، ITS-rDNA، تنوع اللی

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