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Population genetics and gene sequence analysis of *Athelia rolfsii* collected from Northern Iran

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Abstract: *Athelia rolfsii* is a globally dispersed pathogenic fungus, causing white root rot disease in many crops and horticultural plants. In this study, 90 isolates were collected from three provinces of Guilan, Mazandaran and Golestan in northern Iran. Eighteen isolates were selected for sequence analyses based on their host, sampling sites and Mycelial Compatibility Groups (MCG). Translation elongation factor 1-α (*tef1-α*), β-tubulin (*tub2*) genes and rDNA large subunit (LSU) were partially amplified and sequenced in order to conduct phylogenetic analyses. Approximately, all Iranian isolates clustered together in both *tef1-α* and LSU phylogenetic trees. However, after deletion of ambiguous sites, no variations were observed in *tub2* sequences. ISSR and SCoT were also used to investigate the genetic structure of the population. Results of the molecular variance analysis (AMOVA) showed that 70 and 30% of the observed variance corresponded to the difference between and within the populations, respectively. According to these findings, we suggest that mating between populations would be less likely and thus, gene flow is restricted.

Keywords: Genetic diversity, molecular marker, pathogen, phylogeny

INTRODUCTION

Athelia rolfsii (Curzi) C. C. Tu & Kimber (Synonym: *Sclerotium rolfsii* Sacc.) is a monocycle, soil-borne plant pathogen which causes root rot disease on a wide variety of crops, horticultural plants, weeds and forest trees. This fungus can infect around 500 plant species worldwide. The highest infection rate happens in *Fabaceae*, *Solanaceae*, *Cucurbitaceae* members and other vegetables in rotation with beans (Schwartz et al. 2005). Symptoms of the disease include brown to black rot of the root and crown, stem canker and damping-off, knowing as southern blight (wilt) and southern stem rot (Aycock 1966). Different approaches using molecular fingerprinting and sequencing analyses techniques are being applied to investigate population genetics of *Athelia* species. In particular, the application of different markers and subsequently, their relevance to mycelial compatibility groups (MCGs), host and even geographical distribution have been constantly discussed (Harlton et al. 1995, Nalim et al. 1995, Okabe et al. 1998, Cilliers et al., 2000; Okabe & Matsumoto 2000, Punja & Sun, 2001, Alemida et al. 2001).

Recently, restriction digestion analysis of the ITS products has been considered as one of the simplest methods to identify isolates of *Sclerotium* (Prasad et al. 2010). This approach has been developed due to difficulties associated with morphological and physiological identification of *Sclerotium* species (Rasu et al. 2013). In one study, the population of *Sclerotium* causing southern blight disease was effectively differentiated by ITS-RFLP pattern using restriction enzymes *Alu* I, *Hpa* II, *Rsa* I and *Mbo* I, although sclerotial morphology and optimal growth temperature were highly variable and did not match the molecular pattern (Okabe et al. 2000). In another study, Okabe et al. (2001) successfully applied ITS-

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RFLP using restriction enzymes *Alu* I, *Hpa* II, *Rsa* I and *Sau*3AI to unravel the homokaryotic nature of a strain of *A. rolfsii*. In contrast, the genetic diversity of *A. rolfsii* from tropical sugar beet roots was investigated using *Hha* I, *Msp* I and *Pst* I, confirming the heterogeneity among the isolates (Paramasivan et al. 2009). However, it is still a matter of debate whether ITS-RFLP approach reaffirms classical techniques such as mycelial compatibility groups (MCG) (Punja & Sun 2001).

In addition to ITS-RFLP method, the inter simple sequence repeat (ISSR) technique can be counted as an alternative approach to study comparative genetics and analyzing the genetic diversity of *Athelia* species owing to their high level of polymorphism (Moulin et al. 2012). Although Jearai et al. (2017) stated that a set of ISSR markers are well suited for diversity studies compared to random amplification of polymorphic DNA (RAPD), they were both considered as equally informative and efficient DNA marker systems to assess genetic relatedness and diversity among different *Sclerotium* species (Darakshanda et al. 2007, Gawande et al. 2013).

The use of DNA nucleotide sequences in mycology leads to rapid identification and provides the ability to differentiate similar morphological species in the same genus (Sun & Guo 2012).

Common DNA barcodes for fungi include a wide variety of marker genes such as the nuclear internal transcribed spacer (ITS) of the ribosomal RNA genes, the nuclear genes coding for the ribosomal large subunit (*LSU*; 28S rRNA) and the largest subunit of RNA polymerase II (*RPB1*), second largest subunit of RNA polymerase II (*RPB2*), β-tubulin, minichromosome maintenance complex component 7 (*MCM7*), translational elongation factor 1-α (*tef1-α*), γ-actin, sixth subunit of ATP synthase (*ATP6*) and calmodulin (*CAM*). In phylogenetic studies, each of these barcode areas can be used alone or together with different compounds (Tekpinar & Kalmer 2019). The *LSU* region alone or in combination with other genes such as ITS, *SSU*, *tef1-α* and *tub2* have been used by many researchers for phylogenetic studies in fungi (Schroers et al. 2009, Wang et al. 2011, Brown et al. 2014, Asemaninejad et al. 2016, Koch et al. 2017, Guarnaccia et al. 2018, Okabe et al. 2003). Remesal et al. (2012, 2013) used *tef1-α* and *RPB2* genes for phylogenetic analysis of *S. rolfsii* isolates. They suggested that sequencing of *tef1-α* and *RPB2* may be a useful technique for current MCG typing and exploring new MCGs in *S. rolfsii*.

In this study, ISSR and SCoT (Start Codon Targeted) markers were used to study genetic diversity and population genetics of *A. rolfsii* in the southern part of the Caspian Sea region (Northern Iran). Moreover, sequence analyses of the *tef1-α*, *tub2* genes, and *LSU* have also been used to understand the phylogenetic structure and interspecies variation of *A. rolfsii*.

MATERIALS AND METHODS

Sampling, isolation and morphological identification

Ninety isolates of *A. rolfsii* were collected from three provinces (Guilan, Mazandaran and Golestan provinces, 30 isolates per province) in northern Iran during 2011-2016. Small pieces of infected plant crown tissue containing both healthy and infected areas were cut and rinsed. They were disinfected using 0.5% sodium hypochlorite for one min, rinsed with distilled water and transformed to potato dextrose agar (PDA) medium. All procedures were conducted under a laboratory hood. Petri dishes were incubated for 24-48 h under the dark condition at 27°C. The hyphal tip technique was used to transform mycelia to water agar (WA) medium and obtain purified colonies. Sclerotia grown on the surface of colonies were collected and dried under the laboratory hood for two h and finally kept in microtubes at -20°C for the next experiments. Morphological identification was carried out according to Harlton et al. (1995), Punja & Damiani (1996) and Mehri et al. (2020).

DNA extraction

A small mycelial mass of freshly grown colonies on PDA medium from each isolate was transformed to sterilized 1.5 mL microtubes containing 150 mL 5% Chelex solution (Hirata & Takamatsu 1996). They were incubated at 56°C for two h and then placed on the surface of boiling water for eight min. The microtubes were then vortexed for 10-12 s. The latter step was repeated twice. Chelex solution and cell-wall residues were centrifuged for five min at 14000 g at 4°C. The supernatant solution containing extracted DNA was finally transformed to new microtubes and kept at -20°C for sequencing.

Phylogenetic analysis

After considering host, sampling sites and MCGs (Mehri et al. 2020), 18 isolates were selected for detailed phylogenetic analyses (Table 1). Three setts of primers including LR0R/LR5, Btub2Fd/Btub4Rd and EF1-983F/EF1-1567R were used to amplify the large subunit (LSU), beta-tubulin (*tub2*) and translation elongation factor 1 alpha (*tef1-α*) following the protocols of Vilgalys & Hester (1990), Woudenberg et al. (2009) and Rehner & Buckley (2005), respectively, in a Flexible PCR Thermocycler (Analytik Jena, Germany). The resulting sequences were edited and analyzed using MEGA V. 7.0 (Kumar et al. 2016). Sequences were compared with the sequences available in the NCBI GenBank nucleotide database. Several sequences from GenBank were extracted for phylogenetic analyses. Sequence alignment was performed using MUCSLE plug–in of MEGA V. 7.0 with the default settings (Edgar 2004). Phylogenetic trees were obtained using the minimum–evolution (Rzhetsky & Nei 1992) method in MEGA 7.0 (Kumar et al. 2016).

Table 1 Characteristics and accession umbers of the isolates used for sequencing and ITS-RFLP

Isolate	Host Species	Locality	MCG*	Strain	Accession numbers		
					tef1-α	*LSU*	*tub2*
G2P1	*Capsicum frutesens*	Guilan	IRI	IRAN 3669C	MT593183	MT582581	MT593200
G5P1	*Capsicum frutesens*	Guilan	IRI	IRAN 3670C	MT593184		MT593201
G15B1	*Phaseolus vulgaris*	Guilan	IRI	IRAN 3871C	MT593185		MT593202
G17P5	*Capsicum frutesens*	Guilan	IRV	IRAN 3671C	MT593186		MT593203
G19B1	*Phaseolus vulgaris*	Guilan	IRI	IRAN 3672C	MT593187		MT593204
G24S3	*Cucurbita pepo*	Guilan	IRIII	IRAN 3673C	MT593188	MT582582	MT593205
G26B2	*Phaseolus vulgaris*	Guilan	IRII	IRAN 3674C	MT593189	MT582583	MT593206
G27T1	*Solanum lycopersicum*	Guilan	IRI	IRAN 3675C	MT593190	MT582584	MT593207
G28B6	*Phaseolus vulgaris*	Guilan	IRVI	IRAN 3676C	MT593191	MT582585	
O1T8	*Solanum lycopersicum*	Golestan	IRVIII	IRAN 3872C	MT593192	MT582586	
M7B3	*Phaseolus vulgaris*	Mazandaran	IRIII	IRAN 3677C	MT593193		
O18W7	*Chenopodium album*	Golestan	IRVII	IRAN 3678C	MT593194	MT582587	
G29H4	*Helianthus tuberosus*	Guilan	IRIV	IRAN 3679C	MT593195		MT593208
M14B3	*Phaseolus vulgaris*	Mazandaran	IRIII	IRAN 3680C	MT593196	MT582588	MT593209
O22F7	*Helianthus annuus*	Golestan	IRVII	IRAN 3681C			
O30W8	*Amaranthus retroflexus*	Golestan	IRVIII	IRAN 3682C		MT582589	MT593210
O20G3	*Arachis hypogaea*	Golestan	IRIII	IRAN 3684C	MT593198		MT593212
G25O9	*Dicondra rerens*	Guilan	IRX	IRAN 3683C	MT593197	MT582590	MT593211

* MCGs were determined in a previous study (Mehri et al. 2020)

ISSR and SCoT analysis

Methods and part of results of ISSR and SCoT analysis for understanding genetic diversity have been already reported by Mehri et al. (2020). In this study, we used ISSR and SCoT analysis to understand the population genetics of *A. rolfsii*.

These analyses include a set of parameters as follow: percentage of polymorphic loci (P), the numbers of effective alleles (Ne), the numbers of observed alleles (Na), Shannon's information index (I), Nei's gene diversity index (H), the gene diversity statistics, including the total allelic diversity (Ht), the mean allelic diversity within populations (Hs), the proportion of the total allelic diversity found among populations (Gst), Nei's genetic identity and genetic distances between populations, and the gene flow among populations (Nm). All of these statistics were calculated using the software POPGENE (v 1.31). Genetic variation among and within populations were calculated using the phi-statistic through the analysis of molecular variance (AMOVA) with the GenALEX 6.5 software (Peakall & Smouse 2012).

Table 2 ISSR and SCoT primers used for *Athelia rolfsii*

Primer	Sequence 5'-3'	Reference
ISSR		
ISSR1	AGAGAGAGAGAGAGAGT	Tang et al., 2010
ISSR2	AGAGAGAGAGAGAGAGC	Tang et al., 2010
ISSR3	GAGAGAGAGAGAGAGAT	Tang et al., 2010
ISSR4	GAGAGAGAGAGAGAGAC	Tang et al., 2010
ISSR5	ACACACACACACACACG	Yin et al., 2014
ISSR6	TCTCTCTCTCTCTCTCC	Yin et al., 2014
ISSR7	CTCCTCCTCCTCCTCCTC	Yin et al., 2014
ISSR8	GAGAGAGAGAGAGAGAA	Tang et al., 2010
ISSR9	TGCACACACACACAC	Zhao et al., 2013
ISSR10	GTGACACACACACAC	Zhao et al., 2013
ISSR11	GGATGCAAACACACACACAC	Zhao et al., 2013
SCoT		
SCoT1	ACGACATGGCGACCATCG	Zhao et al., 2013
SCoT2	ACCATGGCTACCACCGTG	Zhao et al., 2013
SCoT3	CCATGGCTACCACCGCCA	Zhao et al., 2013
SCoT4	CCATGGCTACCACCGGCG	Zhao et al., 2013

RESULTS

Phylogeny

Due to budget limitations to sequence all isolates, only a part of isolates could be subjected for sequencing analyses. These isolates were selected after our previous cluster analyses so that they represent most MCGs and cover most of the genetic variation among all isolates (Mehri et al. 2020). However, for some isolates, DNA amplification failed due to unknown reasons.

Partial sequences of Elongation factor 1-*a* (*tef1-α*), β-tubulin (*tub2*), and large subunit (LSU) regions were amplified for selected isolates (530, 500 and 970 bp, respectively) to construct phylogenetic trees. Sequences of β-tubulin were not informative and revealed no diversity for isolates. Unfortunately, we did not always obtain successful sequences for the same isolates for both genes. Hence, *tef1-α* and *LSU* were used to generate separate phylogenetic trees. All isolates clustered in one group and, thus, these two genes do not discriminate any MCGs. Although some variation was found for the *LSU* region, it was not sufficient to delimit clearly our Iranian isolates from several other GenBank sequences (Fig. 1). In *tef1-α* phylogenetic trees, our Iranian isolates were more or less separated from most GenBank sequences, except for one isolate (Isolate G28B6). However, this tree did not receive high bootstrap support, probably due to low sequence variations between isolates (Fig. 2). In general, we can conclude that all isolates belong to the same species and there was not, as expected, sufficient variation among our isolates.

Population genetics

Each sampling region was considered as a unique geographic population throughout the analysis. The highest gene diversity and Shannon index were 0.135 and 0.216, respectively, assigned to the Golestan population. In addition, the lowest gene diversity and Shannon index were 0.041 and 0.065, respectively,

assigned to the Mazandaran population. Gene and genotype diversity for the Guilan isolates were 0.116 and 0.201, respectively.

Gene and genotype diversity for the Guilan isolates were 0.116 and 0.201, respectively. The percentage of polymorphic loci varied between 15.87 and 75% (Table 3).

Average total gene diversity (Ht) for all sites, average gene diversity within the population (Hs), average genetic differentiation (Gst), and gene flow (*Nm*) were 0.258, 0.097, 0.623 and 0.303, respectively. Our analysis of molecular variance (AMOVA) revealed that the diversity between populations was relatively high (70%). Thirty percent of the observed variance corresponded to differences among isolates within populations (Table 4). The low variance observed in the isolates within populations indicates their high genetic relatedness. The highest genetic similarities were observed between the Guilan and Mazandaran populations (Table 5).

Fig. 1 Minimum Evolution tree based on *LSU* for 10 taxa of *A. rolfsii*. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA 7. *Pleurotus cystidiosus* was used as an outgroup taxon.

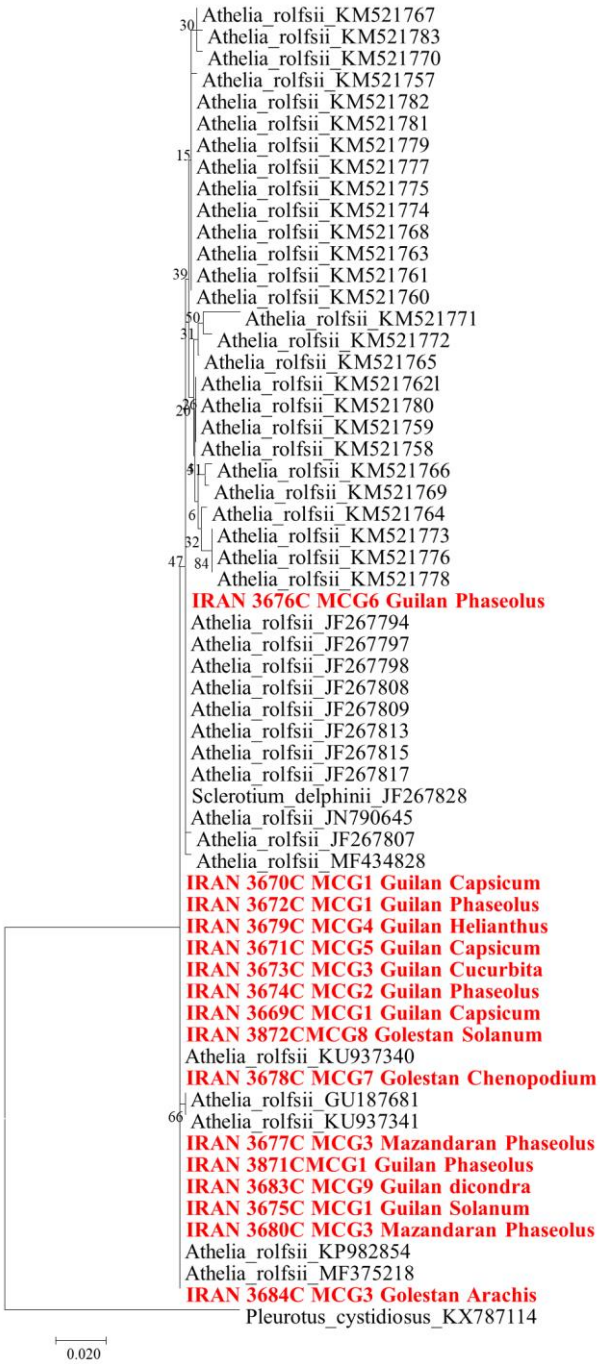

Fig. 2 Minimum Evolution tree based on partial translation elongation factor 1α (*tef1-α*) sequences for 12 taxa of *A. rolfsii*. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA 7. *Pleurotus cystidiosus* was used as outgroup taxon.

Table 3 Analysis of the genetic variation of *Athelia rolfsii.*

Population	Population Size	No. of Effective Alleles	No. of Observed Alleles	Nei's Gene Diversity	Shannon's Information Index	Percentage of Polymorphic Loci
Guilan	30	1.178±0.288	1.75±0.434	0.116±0.147	0.201±0.2	75%
Mazandaran	30	1.065±0.185	1.159±0.366	0.041±0.113	0.065±0.17	15.87%
Golestan	30	1.223±0.344	1.572±0.496	0.135±0.173	0.216±0.242	57.21%
Average	30	1.355±0.273	1.494±0.432	0.097±0.144	0.161±0.204	49%
Total	90	1.430±0.337	1.981±0.138	0.258±0.182	0.394±0.248	98.08

Table 4 Molecular variance analysis results for 90 isolates of *Athelia rolfsii*

Source	df	SS	MS	Estimated Variance	Percentage %
Among Pops	2	1503.311	751.656	24.702	70%
Within Pops	87	920.600	10.582	10.582	30%
Total	89	2423.911	-	35.284	100%

Table 5 Nei's genetic identity (above diagonal) and genetic distance (below diagonal) between populations of *Athelia rolfsii*

	Guilan	Golestan	Mazandaran
Guilan	****	0.6048	0.9901
Golestan	0.5028	****	0.5972
Mazandaran	0.0100	0.5155	****

DISCUSSION

Athelia rolfsii causing root rot disease is considered a serious problem for crops cultivation in contaminated areas due to its wide host range, genetic population diversity, and its ability to produce abundant resistant sclerotia.

In a study conducted by Remesal et al. (2013), seven of the 12 MCGs could be well characterized based on the *tef1-α* region. So far, no study has focused on the genetic variation and population genetics of *A. rolfsii* in Iran. In our study, all isolates did not show any significant differences considering *tef1-α*, LSU and *tub2* sequences. The lack of sufficiently discriminatory power of *tef1-α*, *tub2* and LSU genes indicates a high degree of uniformity of these three genes among Iranian MCGs. Yet, isolate G28B6 (representative for MCG6) was clustered separately in *tef1-α* tree.

Sequence analysis of the amplified products showed a high similarity with *A. rolfsii* sequences from GenBank using BLAST search analysis. This information, based on DNA sequence analysis, indicates that *S. rolfsii* is more prevalent in northern Iran and we could not find any evidence for the occurrence of *S. delphini* in this region.

In the case of ISSR, the area adjacent to the two identical microsatellites in opposite directions is reproduced well. One of the main advantages of this method are its simplicity, reproducibility and high resolution (Zhang et al. 2007). SCoT was first used as a supplement to ISSR in *A. rolfsii*. Zhao et al. (2013) used Scott and ISSR to study the genetic diversity and population structure of *Pleurotus eryngii* var. *tuoliensis*. Generally, a high genetic variation occurs in populations from a wide geographical distribution, sexual reproduction, and extensive ecological niches (James et al. 1999). The highest gene diversity was observed in the Golestan province. High polymorphic bands (PPB) and Nei's gene diversity index (H) are two important parameters that are used to study genetic diversity at the species level (Hamrick & Godt 1989). In this study, polymorphic bands and Nei's gene diversity index were 98.08 and 0.258, respectively, which indicates a high polymorphism in our studied isolates. It has also been shown that the diversity was higher between populations than within them. This finding might imply that the fungus does not sexually reproduce in the region and consequently, spores for a widespread distribution are not available for gene flow between populations. Subsequently, mating between populations would be less likely and thus, gene flow is restricted. As a result, gene exchange between populations and subsequent genetic variation are basically limited to both the mycelial compatibility process and heterokaryon formation. Therefore, identifying dominant MCGs in each region and subsequent investigations on host range and crop race resistance are recommended.

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ژنتیک جمعیت و آنالیز توالی ژنی *Athelia rolfsii* جمع آوری شده از شمال ایران

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چکیده: قارچ *Athelia rolfsii*، یک بیمارگر خاکزی با پراکنش جهانی است که به عنوان عامل پوسیدگی سفید ریشه در بسیاری از گیاهان زراعی و باغی شناخته می‌شود. در این پژوهش تعداد ۹۰ جدایه، از سه استان گیلان، مازندران و گلستان در شمال ایران جمع‌آوری و جداسازی شدند. تعداد ۱۸ جدایه بر اساس میزبان، محل جمع آوری و MCG انتخاب شدند. جهت تجزیه و تحلیل فیلوژنتیک ۱۸ جدایه انتخابی، بخشی از ژن‌های *tef1-α*، *tub2* و *LSU* تکثیر و توالی‌یابی شدند. در درخت‌های رسم شده بر اساس *tef1-α* و *LSU* تمام جدایه‌های ایرانی کنار یکدیگر قرار گرفتند. توالی ژن *tub2* بعد از حذف جایگاه‌های مبهم، فاقد تنوع بود. به منظور ارزیابی ساختار ژنتیک جمعیت های قارچ از نشانگرهای ISSR و SCoT استفاده شد. نتایج تجزیه واریانس مولکولی (AMOVA) نشان داد که ۷۰٪ واریانس به تفاوت بین جمعیت‌ها و ۳۰٪ آن به تنوع درون جمعیتی مربوط بود. لذا آمیختگی جمعیت شانس محتمل نیست و جریان ژنی کمی بین جمعیت‌ها وجود دارد.

کلمات کلیدی: بیمارگر، تنوع ژنتیکی، فیلوژنی، مارکرهای مولکولی

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