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

Z. Mehri
S. A. Khodaparast

Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

A. Aalami

Department of Biotechnology, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

S. Mousanejad
H. Masigol

Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

H. P. Grossart

Department of Experimental Limnology, Leibniz Institute of Freshwater Ecology and Inland Fisheries, Stechlin, Germany

**Abstract:** *Athelia rolfsii* is a globally dispersed pathogenic fungus causing 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 monocyte, 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, known 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, Alemda 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-
RFLP using restriction enzymes Alu I, Hpa II, Rsa I and Sau3AI 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 (tefl-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, tefl-α and tub2 have been used by many researchers for phylogenetic studies in fungi (Schoebers et al. 2009, Wang et al. 2011, Brown et al. 2014, Asemaninejad et al. 2016, Koch et al. 2017, Guarinacci et al. 2018, Okabe et al. 2003). Remesal et al. (2012, 2013) used tefl-α and RPB2 genes for phylogenetic analysis of S. rolfsii isolates. They suggested that sequencing of tefl-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 tefl-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 sets of primers including LR0R/LR5, B tub2Fd/B tub4Rd and EF1-983F/EF1-1567R were used to amplify the large subunit (LSU), beta-tubulin (tub2) and translation elongation factor 1 alpha (tefl-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 MUSCLE 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).
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 Athelia 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).

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 (tefl-a), 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, tefl-a 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 tefl-a 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 geographic population throughout the analysis. 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.

<table>
<thead>
<tr>
<th>Population</th>
<th>Size</th>
<th>No. of Effective Alleles</th>
<th>No. of Observed Alleles</th>
<th>Nei's Gene Diversity</th>
<th>Shannon's Information Index</th>
<th>Percentage of Polymorphic Loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guilan</td>
<td>30</td>
<td>1.178±0.288</td>
<td>1.754±0.343</td>
<td>0.116±0.147</td>
<td>0.201±0.2</td>
<td>75%</td>
</tr>
<tr>
<td>Mazandaran</td>
<td>30</td>
<td>1.065±0.185</td>
<td>1.592±0.366</td>
<td>0.041±0.113</td>
<td>0.065±0.17</td>
<td>15.87%</td>
</tr>
<tr>
<td>Golestan</td>
<td>30</td>
<td>1.223±0.344</td>
<td>1.572±0.496</td>
<td>0.135±0.173</td>
<td>0.216±0.242</td>
<td>57.21%</td>
</tr>
<tr>
<td>Average</td>
<td>30</td>
<td>1.355±0.273</td>
<td>1.494±0.432</td>
<td>0.097±0.144</td>
<td>0.161±0.204</td>
<td>49%</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>1.430±0.337</td>
<td>1.981±0.138</td>
<td>0.258±0.182</td>
<td>0.394±0.248</td>
<td>98.08%</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>Estimated Variance</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among Pops</td>
<td>2</td>
<td>1503.311</td>
<td>751.656</td>
<td>70%</td>
</tr>
<tr>
<td>Within Pops</td>
<td>87</td>
<td>920.600</td>
<td>10.582</td>
<td>30%</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>2423.911</td>
<td></td>
<td>100%</td>
</tr>
</tbody>
</table>
Table 5 Nei’s genetic identity (above diagonal) and genetic distance (below diagonal) between populations of *Athelia rolfsii*

<table>
<thead>
<tr>
<th></th>
<th>Guilan</th>
<th>Golestan</th>
<th>Mazandaran</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guilan</td>
<td>0.5028</td>
<td>0.6048</td>
<td>0.5972</td>
</tr>
<tr>
<td>Golestan</td>
<td>0.6048</td>
<td>0.5028</td>
<td>0.5155</td>
</tr>
<tr>
<td>Mazandaran</td>
<td>0.5972</td>
<td>0.5155</td>
<td>0.5028</td>
</tr>
</tbody>
</table>

**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 is 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.

**ACKNOWLEDGEMENTS**

This work was supported in part by a grant from the Deputy of Research and Technology of the University of Guilan, Iran and the German Science Foundation project GR1540/33-1 to HPG.

**REFERENCES**


Hamrick JL, Godt MW. 1989. Allozyme diversity in plant species. In: Plant population genetics, breeding, and genetic resources (Brown A HD,


ژنتیک جمعیت و آنالیز توالی زنی Athelia rolfsii جمع‌آوری شده از شمال ایران

چکیده: قارچ Athelia rolfsii یک بیمارگر خاکزی با پراکنش جهانی است که به عنوان عامل عامل پوسیدگی سفید ریشه در بسیاری از گیاهان زراعی و باغی شناخته می‌شود. در این پژوهش تعداد 90 جدایه از سه استان گیلان، مازندران و گلستان در شمال ایران جمع‌آوری و جداسازی شدند. تعداد 18 جدایه بر اساس میزبان، محل جمع‌آوری و MCG انتخاب شدند. جهت تجزیه و تحلیل فیلوژنیک 18 جدایه انتخابی، به‌کار رفت. درخت у نتیجه این تجزیه، این جدایه‌ها با یکدیگر کنار قرار گرفتند. توالی ژن tub2 بعد از حذف جایگاه‌های مبهم، فاقد تنوع بود. به منظور ارزیابی ساختار جمعیت قارچ از نشان‌گران SCoT و ISSR استفاده شد. نتایج تجزیه واریانس مولکولی (AMOVA) نشان داد که 20% واریانس به تفاوت بین جمعیت‌ها و 80% آن به تنوع درون جمعیتی مربوط بود. لذا آمیختگی جمعیت شناس محتمل نبود و جریان زنی کمی بین جمعیت‌ها وجود دارد.

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

Email: khodaparast@guilan.ac.ir
مکاتبه کننده: سید‌اکبر خداپرست
تاریخ دریافت: 1398/11/13
تاریخ پذیرش: 1399/02/18