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Original Article

بررسی تنوع ژنتیکی جدایه های *کالونکتریا سودوناویکولاتا* عامل بیماری بلایت شمشاد در جنگل های هیرکانی با استفاده از نشانگرهای RAPD و ISSR

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چکیده

سابقه و هدف: شمشاد گیاه بومی ایران بوده که اهمیت خاصی در میان ذخایر جنگلی جهان دارد و در سرتاسر جنگل های هیرکانی گسترش دارد. در سال های اخیر *کالونکتریا سودوناویکولاتا* یکی از مهمترین علت های بلایت و ریزش برگ های شمشاد در ایران بوده است. این مطالعه برای اولین بار در ایران با هدف بررسی تنوع ژنتیکی جدایه های *کالونکتریا سودوناویکولاتا* در جنگل های هیرکانی با استفاده از نشانگرهای RAPD و ISSR انجام شد.

مواد و روش ها: در این مطالعه تعداد ۷۵ جدایه قارچ از شمشاد در جنگل های هیرکانی شمال ایران جداسازی شد. جدایه ها از نظر ریخت شناسی و رنگ کلنی بررسی شدند. تنوع ژنتیکی جدایه ها با استفاده از نشانگرهای RAPD و ISSR مورد مطالعه قرار گرفت. بخشی از ژن بتاتوبولین در جدایه های *کالونکتریا سودوناویکولاتا* تعیین توالی شد و در NCBI ثبت گردید. آنالیز فیلوژنتیکی به کمک نرم افزار PAUP انجام شد.

یافته ها: کلنی ها بر روی محیط PDA دارای مشخصاتی مانند رنگ قهوه ای با هاله کمرنگ، کنیدی های استوانه ای و در دو انتها دایره ای شکل، تک دیواره با قطر ۶۸-۴۸ میکرومتر، استیپ بلند به طول ۹۰-۱۴۰ میکرومتر، دارای جداره عرضی و در انتها دارای وزیکل بیضی شکل دارای و پاپیل در نوک بودند. درخت فیلوژنتیکی رسم شده بر اساس ژن بتا توبولین، نشان داد که تمامی جدایه ها در یک گروه قرار دارند. بررسی تنوع ژنتیکی نشان داد که جمعیت جدایه های این قارچ در ایران از تنوع ژنتیکی پایینی برخوردار هستند.

نتیجه گیری: به نظر می رسد که در برخی از مناطق با توجه به پدیده فشار انتخاب، تغییر ژنتیکی در عامل بیماری آغاز شده باشد.

واژگان کلیدی: ژن بتا توبولین، *کالونکتریا سودوناویکولاتا*، نشانگر ISSR، نشانگر RAPD.

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Original Article

Study on genetic variation of Hyrcanian *Calonectria pseudonaviculata* (boxwood blight agent) isolates using RAPD and ISSR markers

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Abstract

Background & Objectives: Boxwood tree is one of the Iranian endemic trees expanded throughout Hyrcanian forests. They are of particular importance among the world's forest reserves. Recently, *Calonectria pseudonaviculata* has been considered as one of the most important causes of blight and leaf defoliation of the boxwood in Iran. For the first time, the present study was aimed to evaluate the genetic variation of *Hyrcanian C. pseudonaviculata* isolates using RAPD and ISSR markers.

Material & Methods: In this study, 75 fungal isolates were collected from the infected boxwoods throughout Hyrcanian forests in the North of Iran. The isolates were assessed based on morphology and colony color. Genetic diversity of the isolates was studied using RAPD and ISSR markers. A part of the beta-tubulin gene was sequenced and deposited at NCBI. Phylogenetic analysis was carried out using PAUP* v. 4.0b10.

Results: The colony color of isolates on potato dextrose agar (PDA) medium was brown with pale hale, conidia were cylindrical, rounded at both ends, 1-septate, 48- 68 µm. Stipe was long (90-14 µm) and hyaline, with the extension terminating in a broadly ellipsoid papillate vesicle, the widest part above the middle. Phylogenetic tree based on the β-tubulin gene showed that all isolates are placed into the same group. Our results indicated that the population of this fungus has a low genetic diversity in Iran.

Conclusion: It seems that the variation of this pathogen is started to change genetically in some areas due to selection pressure phenomenon.

Keywords: β-tubulin gene, *Calonectria pseudonaviculata*, ISSR marker, RAPD marker.

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Introduction

Boxwood blight caused by *Calonectria pseudonaviculata* is one of the most severe diseases of Boxwood plants. The symptoms of the disease are severe dieback and defoliation. The fungus was first found in the United Kingdom (UK) in 1994 causing blight disease on *Buxus* species, and after that, the disease was considered to be widespread throughout Europe (1).

C. pseudonaviculata was first reported in Iran Hyrcanian forest in 2012 and in Mazandaran and Guilan forests in 2013 (2-4). The disease has rapidly spread throughout Hyrcaian forests and caused extensive damage, but it was discovered and reported at the end of Hyrcanian forest in Eastern Golestan province, two years later (5).

Recently, boxwood tree decline has occurred in 40,000 hectares of Hyrcanian forest in the north of Iran (6). Reported symptoms included root rot, stem lesions, stem cankers, dieback, and leaf spots (1). The pathogen has a rapid disease cycle that can be completed within one week; infection can spread quickly in warm, humid conditions (7).

Many polymerase chain reaction (PCR)-based approaches have been reported to study genetic relationships in fungi (8). RAPD and ISSR are among common techniques (9, 10). ISSR analysis has yielded vital and reliable molecular markers for studies focusing on population genetics as well as the evolutionary biology of many eukaryotes including plant pathogenic fungi (11).

Henricot and Culham (2002), sequenced β-tubulin, ITS, and MAT1-2 genes to better understand the phylogeny of *Calonectria*. Amplified fragment length polymorphism (AFLP) fingerprinting was used to identify genetic variation among 18 *C. pseudonaviculata* isolates from different geographic regions. AFLP analysis showed little genetic variation among the isolates (1, 7).

In another study, multilocus phylogenetic analysis of a collection including 234 *Calonectria* isolates from 15 countries and four continents indicated two genetic clades including G1 and G2. The isolates in G1 and G2 clades were identified as *C. pseudonaviculata sensu stricto,* and *Calonectria henricotiae* sp. nov., respectively (12). Aside from studies in the U.K. and New Zealand, there have been very few studies on genetic analysis of *C. pseudonaviculata*, throughout the world including Iran.

This study was conducted to better understand the population genetic and diversity of Hyrcanain forest C. *pseudonaviculata* isolates, using partial β-tubulins sequencing, as well as ISSR and RAPD molecular markers.

Material and Methods

Fungal isolates and morphological characterization

Seventy-five isolates were collected from diseased boxwood plants (*Buxus sempervirens* subsp. *Hyrcana)* from Hyrcaian forest of Guilan, Mazandaran, and Golestan throughout all different areas, with different altitudes and climates regions.

The infected plant tissues (necrotic spots on the leaves and black streaks on the stems) were surface sterilized using hypochlorite sodium (1%), cultured on PDA (Merck, Germany) medium, and incubated at 25 °C for a week till

Table 1. The characters of the RAPD primers.

Primers	Sequence (5´-3´)	polymorphic information content (PIC)	Number of polymorphic bands	Annealing temperature
D7	5´-CGG CCA CCG T-3´	0.89	6	34 °C
289	5´-ATC AAG CTGC-3´	0.87	4	32 °C
M13	5´-GGT GGT CAA G-3'	0.85	5	32 °C
208	5´-ACG GCC GAC C-3´	0.85	5	34 °C
285	5´-CCG GCC TTA G-3´	0.90	6	33 °C
215	5´-ACC GGG TTT C -3´	0.80	3	32 °C

the isolation the fungal causal agent. Single spore isolation of the cultures was made on water agar (Merck, Germany).

The morphology and growth rate of the colonies were analyzed both on PDA and malt extract agar (MEA) at 25 °C for 10 days(18). For spore production, the isolates were grown on PCA (carrot pieces 20 g, potato pieces 20 g, agar 20 g per liter) under near-UV light at 25°C. Morphological characteristics including length, shape and the number of septa of conidia, also length and the vesicle characterization of stipe were observed using photomicroscopy and measured using image analyzer system (Leica Qwin) for 40 observations per structure (13).

DNA extraction

The pure culture of the fungus was grown on PDA medium at 25 °C in the incubator for 7 days. The mycelia mat was harvested and crushed to powder in pre-chilled pestle and mortar in liquid nitrogen. DNA was extracted by CTAB method (14). DNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Isogen Life Science).

Amplification and sequencing of β-tubulin DNA

Twenty representative *C. pseudonaviculata* isolates were selected based on variation in colony patterns, a growth rate in optimum temperature, and height above sea level (Table 2). The β-tubulin sequences of isolates were amplified using bt2a and bt2b primers (15) in a 25 µl reaction volume containing 2.5 µl 10XPCR buffer (Fermentas, Germany), 0.5 µl of dNTP, 0.75 µl $MgCl_2$, 1 µl of each primer, 0.25 U *Taq* DNA Polymerase (Fermentas, Germany), and 50 ng genomic DNA. DNA amplification was performed with a BioRad thermo-cycler, with an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 30 s, 72 °C for 30 min, and a final extension at 72 °C for 5 min (12).

Amplification products were separated by 1.2% agarose gel electrophoresis, and the bands were observed with the addition of 10,000X Sybr Safe DNA Gel Stain under UV light on a Gel Doc 1000 system (Bio-Rad, UK). Amplicons were sequenced in both directions using the Sanger sequencing method at Macrogen (Seoul, Korea). The obtained sequences have been deposited in GenBank.

Tubulin sequence analysis

The nucleotide sequences were initially aligned along with data retrieved from GenBank using the multi-alignment program CLUSTAL X (16). The MP analysis was performed with PAUP v. 4.0b10 (17). The phylogenetic tree of β-tubulins of 20 isolates was inferred using heuristic search option with tree-bisection-reconnection (TBR) branch swapping, and 1,000 random sequence

Table 2. Characteristics of 20 sequenced isolates of *C. pseudonaviculata.*

Isolate code	ISSR group	RAPD group	GenBank accession number
MC100	G2	G1	KY065193
MC102	G2	G1	KY065201
MR101	G1	G1	KY065194
GR102	G2	G1	KY065206
MD100	G1	G1	KY065195
MK104	G1	G1	KY065196
MK-105	G1	G1	KY065191
MV101	G3	G1	KY065199
MG101	G3	G1	KY065200
MN107	G3	G4	KY065202
ML 105	G2	G1	KY065203
MA101	G1	G1	KY065204
MCh102	G3	G2	KY065205
MM102	G1	G1	KY065208
MT101	G1	G1	KY065198
GA402	G1	G1	KY065190
GA408	G1	G3	KY065189
GK605	G1	G1	KY065192
GB104	G1	G1	KY065207
LB102	G2	G1	KY065197

additions. The robustness of the equally most parsimonious trees was evaluated by 1,000 bootstraps.

β-RAPD analysis of isolates

Six oligomer primers were tested in RAPD-PCR to evaluate the genetic diversity among 75 *C. pseudonaviculata* isolates (Table 1). PCR reactions (25 µl) contained 50 ng genomic DNA, 1.5 µl of each primer (10 pmol/ml), and 12.5 µl Dream Taq PCR Mastermix (Fermentas, Germany), which were amplified in a BioRad thermocycler. Reaction conditions consisted of an initial denaturation step at 94 ºC for 3 min, followed by 35 cycles at 94 ºC for 2 min, annealing temperature for 1 min (Table 1), and 72 ºC for 1.5 min, and then a final extension step at 72 ºC for 10 min (10). Fragment analysis was performed on 2 % agarose gels and the bands were observed with the addition of 10,000X SybrSafe DNA Gel Stain under UV light on a Gel Doc 1000 system (Bio-Rad, UK).

PIC values of each primer were calculated using PIC= $\frac{N(1-\sum pi^2)}{n-1}$ formula.

ISSR analysis of isolates

Eight ISSR primers which had been used for *Pseudonectria buxi* ISSR experiments (18) were used here to screen the genetic variation: (CAG) 6CC, DD (CCA)5, (ACA)5, CCA (TGA) 5TG, (CAA)5, (AG)8, (ACC) 6CCA, and (CAC)5. The primers annealing temperatures were tested with a gradient thermal cycler (Bio-Rad Thermal Cycler), with temperatures ranging from 45 to 56 °C to find the optimal annealing temperature to achieve clear banding patterns. The reactions were done in a total volume of 25 µl containing 2.5 µl 10x PCR buffer (50 mm Tris-HCl, pH 8.5), 0.5 µl dNTP (10mm), 0.75µl $MgCl_2$ (50 mm), 1 µl primer (10 pmol/ml), 0.25 µl U*Taq* DNA Polymerase (5 unit/ml), and 2 µl DNA. DNA amplification was performed with a BioRad thermocycler, with an initial denaturation step of 94 °C for 1.5 min, followed by 35 cycles of 94 °C for 2 min, 56 °C for 1 min, 72 °C for 1.5 min, and a final extension at 72 °C for 10 min (21).

The fragment analysis was performed on 2% agarose gels, and the bands were observed with the addition of 10,000X SybrSafe DNA Gel Stain under UV light on a Gel Doc 1000 system (Bio-Rad, UK).

Data analysis and Scoring

The data were scored as the presence (1) or absence (0) of the individual band for each *C. pseudonaviculata* isolate in RAPD-PCR and ISSR-PCR. Data analysis was computed by NTSYSpc-2.02e, and the resulting similarity

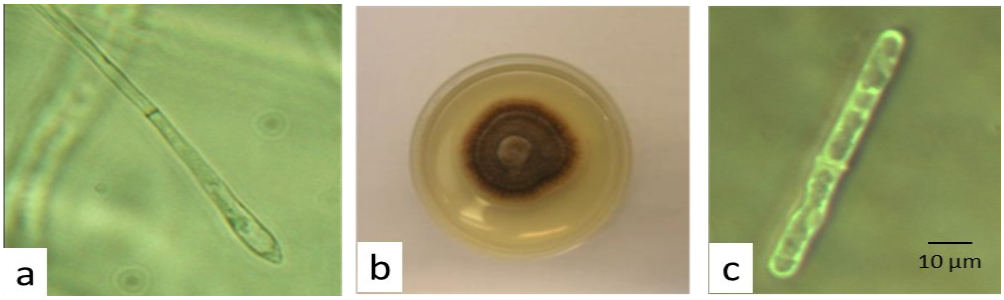

Fig. 1. a: stip, b: colony pattern of *C. pseudonaviculata* on MEA in 25 °C following seven days. c: conidium.

matrix was used for unweighted pair group method with arithmetic mean (UPGMA).

Results

Morphological characteristics

All 75 isolates were identified as *C. pseudonaviculata* based on morphological characteristics (Table 3) by having one-septate conidia and ellipsoidal vesicles with papillate apices (19, 20). The colony color on MEA was brown with pale hale, conidia were cylindrical, rounded at both ends, 1-septate, 48-68 (47.3)×4-6 (4.8) µm. Stipe was long (90-14 µm) and hyaline, with the extension terminating in a broadly ellipsoid papillate vesicle, the widest part above the middle.

β-tubulin sequences

bt2a and bt2b primers amplified a segment of the β-tubulin gene that includes introns three, four and five. Primers amplified the expected 350 bp region in 20 representative isolates. The trimmed β-tubulin sequence of the isolates was matched about 99 to 100 percent of the registered sequences of *C. pseudonaviculata* in NCBI. The obtained sequences have been deposited in GenBank under accession numbers KY065189– KY065208 (Table 2), and the phylogenetic trees were constructed using another 12 sequences obtained from GenBank, with *Cylindrocladiella peruviana* as an out-group taxon. Phylogenetic trees generated by parsimony methods on the aligned β-tubulin dataset had similar topologies for *C. pseudonaviculata* (Figure 2).

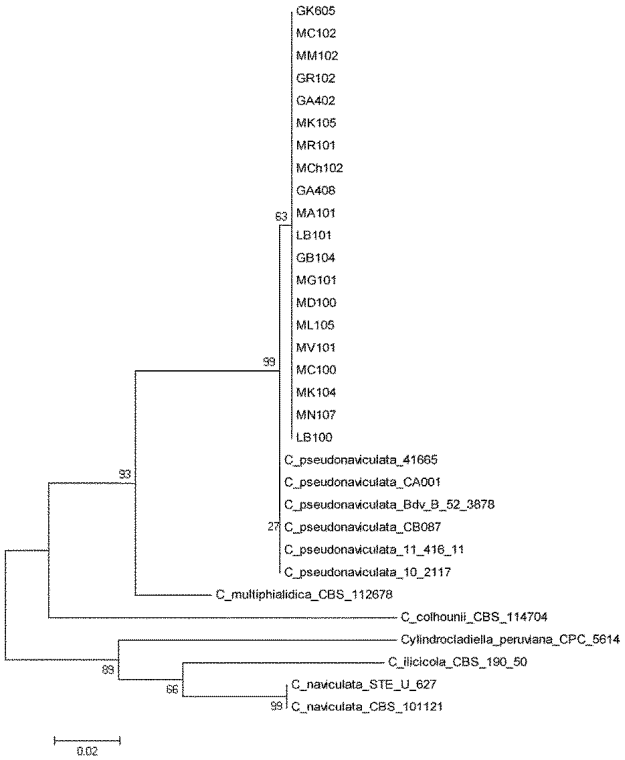

Fig. 2. Phylogenetic tree of β-tub sequences of *C. pseudonaviculata* species given from NCBI and our isolates. The trees are drawn to scale, with branch lengths measured by the number of substitutions per site. The Maximum parsimony values are given at the nodes.

Table 3. Isolate code and the origin of *C. pseudonaviculata.*

Isolate Code	Origin (Province-Location)	Isolate Code	Origin (Province-Location)
MC-100	Mazandaran-Sisangan	MA-104	Mazandaran-Afrachal
MC-101	Mazandaran-Sisangan	MA-105	Mazandaran-Afrachal
MC-102	Mazandaran-Sisangan	MV-101	Mazandaran-Savadkooh/Vachat
MC-103	Mazandaran-Sisangan	MCh-101	Mazandaran-Chaeebagh
MC-104	Mazandaran-Sisangan	MCh-102	Mazandaran-Chaeebagh
MC-105	Mazandaran-Sisangan	MK-101	Mazandaran-Sorkhkola
MR-100	Mazandaran-Chalandar	MK-102	Mazandaran-Sorkhkola
MR-101	Mazandaran-Chalandar	MK-103	Mazandaran-Sorkhkola
MD-100	Mazandaran-Kohnesara	MK-104	Mazandaran-Sorkhkola
MD-101	Mazandaran-Kohnesara	MK-105	Mazandaran-Sorkhkola
MD-102	Mazandaran-Kohnesara	MK-106	Mazandaran-Sorkhkola
MT-100	Mazandaran-Toskatook	MK-107	Mazandaran-Sorkhkola
MT-101	Mazandaran-Toskatook	LB-101	Golestan-Bandargaz
MT-102	Mazandaran-Toskatook	LB-102	Golestan-Bandargaz
MM-101	Mazandaran-Mashelak	LB-103	Golestan-Bandargaz
MM-102	Mazandaran-Mashelak	LB-104	Golestan-Bandargaz
ML-101	Mazandaran-Liresar	LB-105	Golestan-Bandargaz
ML-102	Mazandaran-Liresar	GA-400	Guilan-Astara
ML-103	Mazandaran-Liresar	GA-402	Guilan-Astara
ML-104	Mazandaran-Liresar	GA-403	Guilan-Astara
ML-105	Mazandaran-Liresar	GA-404	Guilan-Astara
ML-106	Mazandaran-Liresar	GA-405	Guilan-Astara
MG-101	Mazandaran-Gisa	GA-408	Guilan-Astara
MG-102	Mazandaran-Gisa	Gk-608	Guilan-Kalat
MG-103	Mazandaran-Gisa	GK-602	Guilan-Kalat
MS-101	Mazandaran-Sari	GK-603	Guilan-Kalat
MS-102	Mazandaran-Ghaemshahr	GK-605	Guilan-Kalat
MN-101	Mazandaran-Neka	GK-606	Guilan-Kalat
MN-102	Mazandaran-Neka	GK-607	Guilan-Kalat
MN-103	Mazandaran-Neka	GS-102	Guilan-Siyahkal
MN-104	Mazandaran-Neka	GS-106	Guilan-Siyahkal
MN-105	Mazandaran-Neka	GS-107	Guilan-Siyahkal
MN-106	Mazandaran-Neka	GM-104	Guilan-Gisoom
MN-107	Mazandaran-Neka	GM-103	Guilan-Gisoom
MA-101	Mazandaran-Afrachal	GB-104	Guilan-Bijarkenar
MA-102	Mazandaran-Afrachal	GB-105	Guilan-Bijarkenar
MA-103	Mazandaran-Afrachal	GR-104	Guilan-Roodsar
		GR-101	Guilan-Roodsar

C. pseudonaviculata isolates which were collected from boxwood trees of Mazandaran, Guilan, and Golestan Hyrcanian forests were considered as one group by 99 bootstrap supporting. No polymorphism was found among the isolates *btub* sequences.

RAPD-PCR

All six selected primers showed good polymorphism for the studied isolates and had high PIC values ranging from 0.8 for primer No. 215 to 0.9 for primer No. 285 (Table 1). The sizes of obtained bands were estimated about 200-3000 bp. Seventy-five bands were obtained among which 28 bands were

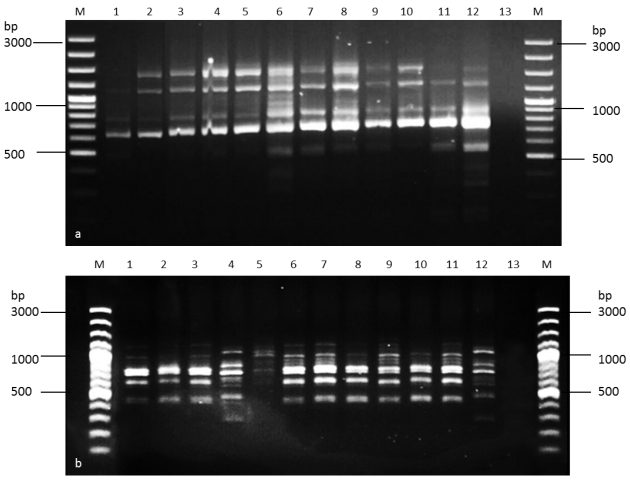

Fig. 3. RAPD pattern of (a) 285; (b) 208. Lane 1: MCh101, lane 2: MG101, lane 3: LB101, lane 4: MN104, lane 5: GA408, lane 6: MT100, lane 7: MD101, lane 8: MA102, lane 9: MC100, lane10: ML102, lane 11: MM102, lane 12: GK603, lane 13: blank. Marker: SM0323.

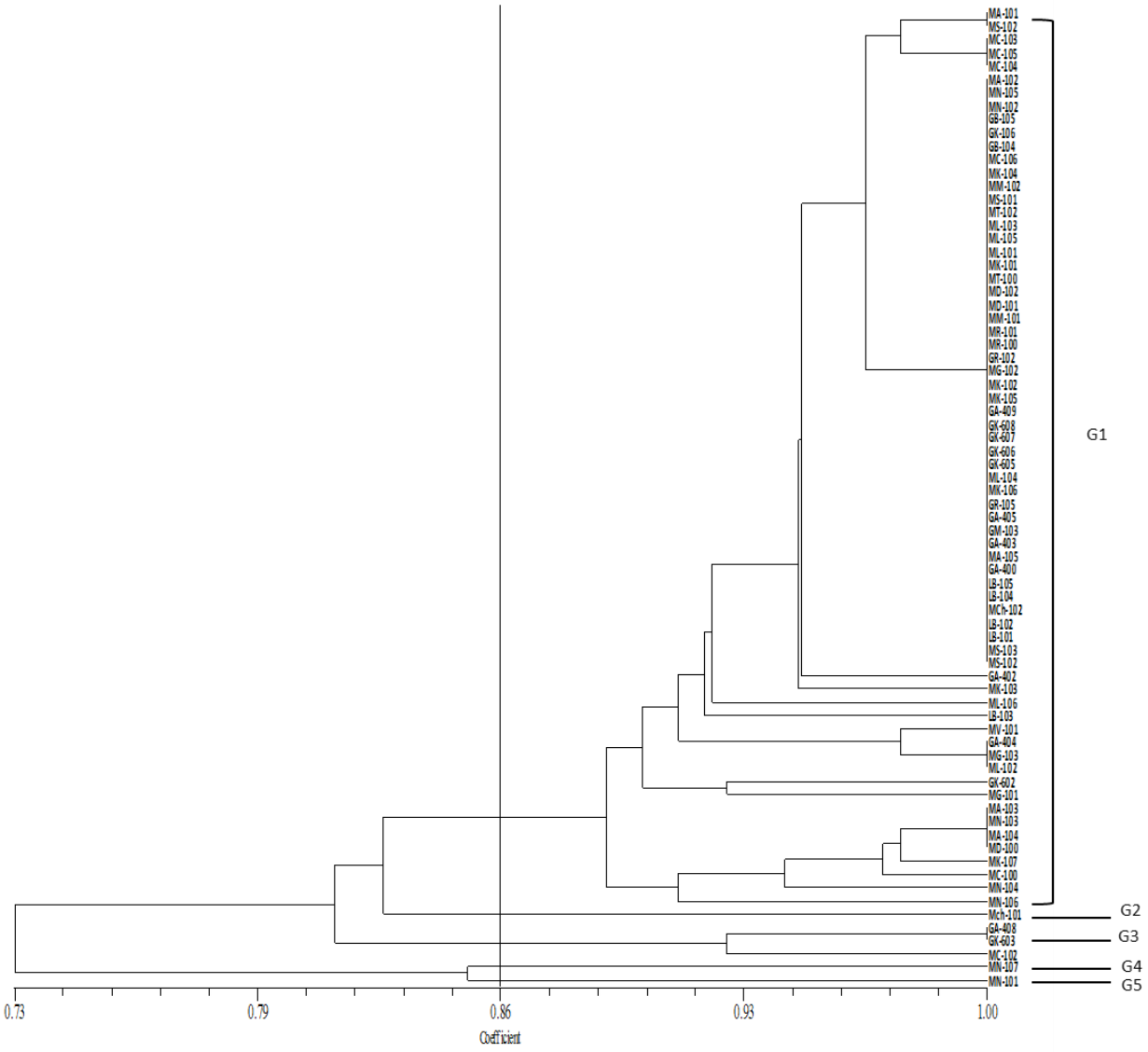

Fig. 4. Cluster analysis of RAPD patterns of *C. pseudonaviculata* isolates collected from different areas of Hyrcanian forest, using the UPGMA algorithm of the NTSYS–Pc v. 2.0.

polymorphic (Figure 3). According to the cluster analysis, isolates formed five cluster groups with 86% genetic similarity level (Figure 4). The majority of isolates (69 isolates) were placed in group G1 including isolates belonging to Mazandaran, Guilan, and Golestan provinces.

Group G2 with only one isolate belonged to Mazandaran province. Group G3 was divided into two subgroups including one subgroup with two isolates collected from the same area of Guilan province, and the other subgroup with one isolate collected from North West of Mazandaran (Sisangan). The hallmarks of this study were group G4 and G5, which both had one member that was collected from the same region (Neka). These two isolates seemed ougroup related to other isolates. The results of RAPD-PCR showed low genetic diversity among Iranian isolates used in this study.

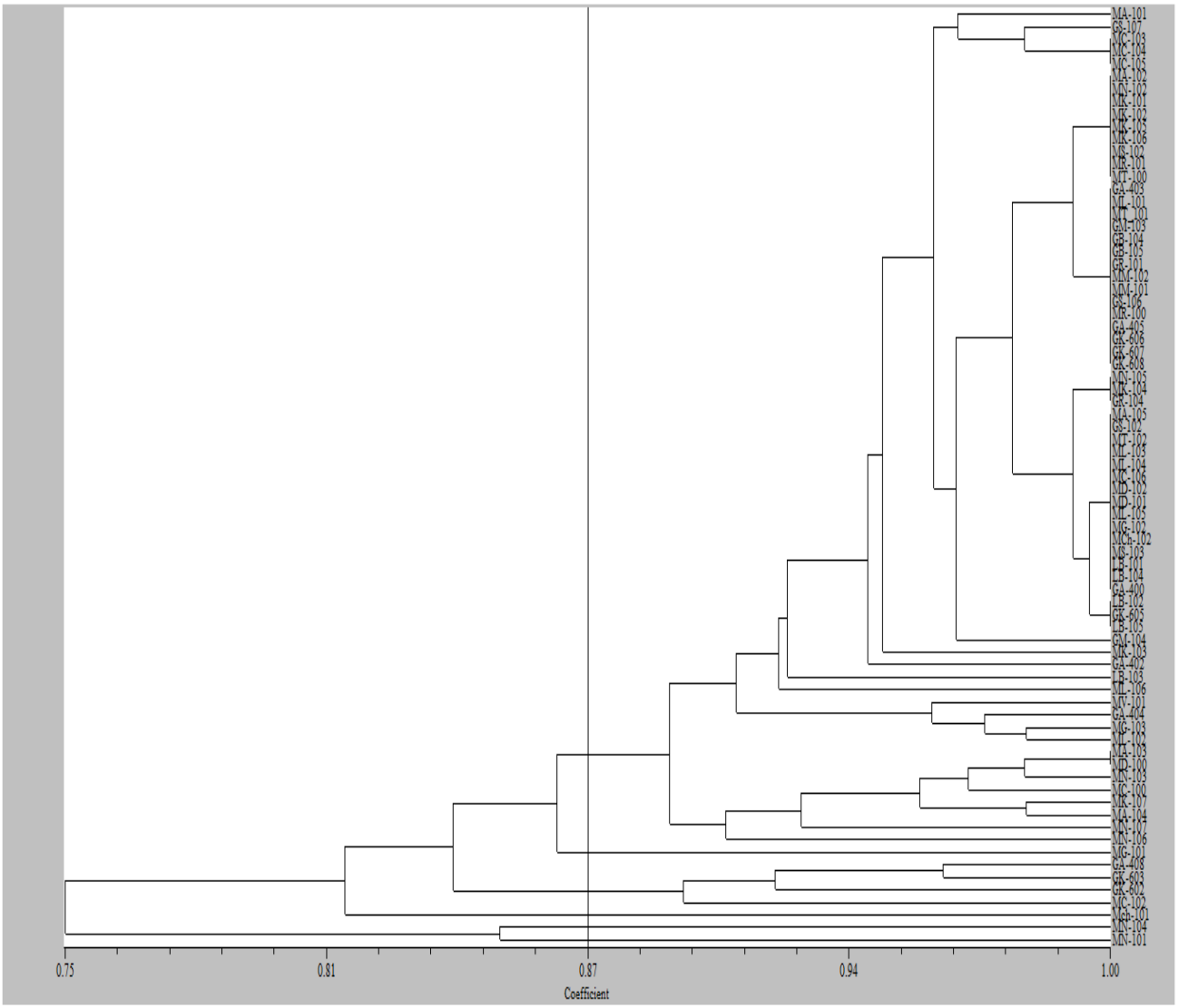

Fig. 5. Integrated inter-simple sequence repeat and random amplified polymorphic DNA dendrogram showing relationships among *Calonectria pseudonaviculata* isolates. Similarity coefficient was calculated by the method of Dice_s. A dendrogram was constructed from the similarity coefficients by using unweighted pair-grouped method.

The RAPD-PCR method could not separate isolates according to their collected locations, morphological differences or violence level.

ISSR-PCR analysis

Seventy-five isolates, used for RAPD analyses were tested with 8 ISSR primers. Two of these primers including (ACC)6 CCA and (CCA)5 produced clear scorable bands, but the other ones did not amplify any bands. The primer (ACC) 6CCA amplified an average of 8 fragments with the length of 200 and 3000 bp, where the highest number of fragments produced was 8. Cluster analysis of ISSR fingerprints classified 75 isolates into three major groups at an arbitrary level of 90% similarity.

Moreover, combined dendrogram of RAPD and ISSR datasets was observed to get a similar distribution among *C. pseudonavicula-*

ta isolates with about 87% similarity as individual analysis (Figure 5). Group1 formed the largest group with 66 isolates from 3 provinces. The remaining groups included Group 2 (MG101/Mazandaran-Gisa), Group 4 (Mch101/Mazandaran-Savadkooh), Group 5 (MN104/ Mazandaran-Neka), and Group 6 (MN101/ Mazandaran-Neka) with one isolate, and group 3 with four isolates from Mazandaran and Guilan provinces.

Discussion

To test the genetic variation among the isolates from different geographic regions, 8 ISSR and RAPD markers were screened with isolates collected from Mazandaran, Guilan, and Golestan. Henricot and Culham (2002) used AFLP to assess if there are any genetic differences between *C. pseudonaviculata* isolates from the UK and from New Zealand (1). At that time, these were the only countries where *C. pseudonaviculata* had been reported. The isolates were genetically homogenous and considered to be a clonal population. Since then, *C. pseudonaviculata* has been officially confirmed in nearly 20 countries. It was unclear if all these observations were caused by international movement of one clonal lineage, or if additional introduction (s) from an unknown center of origin have occurred during the past decade, as was the case for *Phytophthora ramorum* (21, 22).

To address this question, in 2016 a study was performed by Gehesquiere et al to determine the genetic diversity among a larger international collection of *C. pseudonaviculata* isolates using AFLP method (12). Their results showed that the multilocus sequencing strongly supports AFLP data. No sequence difference was observed among *C. pseudonaviculata* isolates. In another study in Canada, ISSR marker was used to detect the variation between the Canadian and Europe isolates. While no genetic variation or polymorphisms were seen for any of the isolates from Ontario. A polymorphism was found in the isolate from Germany (23).

A number of polymorphic markers have been reported for *Cylindrocladium parasiticum* and *C. pauciramosum* (24, 25). These markers have been tested against several other *Cylindrocladium* species, but not against *C. buxicola,* as indicated by literature survey. In thepresent study, the analysis of 75 isolates using RAPD and ISSR markers showed low genetic diversity among Iranian isolates. Our results showed that all isolates are genetically homogeneous. On the other hand, β-tubulin partial sequencing of isolates showed that all Iranian isolates of *C. pseudonaviculata* are placed in one group close to the other countries' isolates. Our results are consistent with another study in which β-tubulin sequencing of 234 *C. pseudonaviculata* isolates from 15 countries and 4 continents placed all isolates in one group namely *C. pseudonaviculata sensustricto*, with no genetic diversity among the isolates of this clade (12). Comparing our results with those of other countries showed that polymorphism was found in European isolates as the first place that the causal agent was observed (23).

Conclusion

In conclusion, it seems that the limited genetic diversity among Iranian isolates is probably

due to an interaction between the pathogen and its specific host which is limited to one variety namely *B. sempervirens*var.

Hyrcana has been growing in Hyrcanian forests as the endemic plant for thousands of years. It has been locally regenerated and showed less genetic plant diversity. It also has been resulted in lack of playing a role of host pressure in the pathogen.

The results of this study showed that the genetic population of this pathogen is started to change in some areas. For example, the isolate MN107 and MN101 collected from Neka, Mazandaran which is placed as one separate group in RAPD and ISSR analysis, may indicate the start of a divergence. In order to prove the accuracy of this hypothesis, we need more genetic data on this fungus population throughout Iran.

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