International Journal of Biological Research, 11 (2) (2024) 79-85

International Journal of Biological Research

Website: www.sciencepubco.com/index.php/IJBR

Research paper

Comparison of ISSR indicators in distinguishing aspergillus fumigatus isolates from different sources

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Abstract

After the samples are obtained, the research's goal is to determine A. fumigatus, the fungus that causes disease and a severe type of pneumonia that can be fatal. This method entails processing DNA with precise and balanced amounts of primers. As a result, after the migration process ended, suitable images were obtained, which served as a helpful reference for differentiating the isolates. If the band was cut from the gel after staining, more single bands with particular molecular weights emerged to benefit from those bands by their dimensions for research and other studies. Furthermore, some of the pioneers of this approach can discern between medical and environmental isolates. ISSR4 effectiveness has been shown in ISSR4 and proved that it's efficient in identifying between the first medical isolate, which has two bands with a molecular weight of 500bp, and yet another of 350bp.In contrast, he observed single bands in the seven environmental isolates that were connected to a weight of 250. In contrast to his companion, ISSR3, who was unable to distinguish that group of isolates with distinctive single bands, it was determined that he was a special baddie in that he was able to distinguish both isolates with distinct weights and a polymorphism card equal to 100 with high efficiency. In opposition to ISSR 9, the joke in that group's initiator, which is superior to the remainder of the group, ISSR 5 is exceptionally fragile. Concerning a total of five independent packages for medical isolation, each of which has an efficiency of one and a polymorphism of 100 within the molecular weight range of 900, 600, 350, and 250 bp. He was able to accurately differentiate across the two isolates utilizing a molecular weight difference of 200bp. for one packet of the isolate derived from river water.

Keywords: *Aspergillus Fumigatus; Zea Mays; Aspergillosis; Phenotypically; ISSR.*

1. Introduction

Studying genetic diversity in the first place is necessary and important in sustainable development, as it provides an opportunity to identify genetic groups, whether old or newly developed. Therefore, it gives a greater opportunity to distinguish fungi in terms of treatment and resistance. Aspergillosis is considered one of the causes that threaten not only people with weak immunity but also those who accompany them, as well as those who are hospitalized. Therefore, its diagnosis and treatment are necessary to limit its spread [19] Since Aspergillus spreads rapidly, it contains small, lightweight spores that travel easily through the air and cause severe invasiveness [18] Aspergillosis fumigatus is a serious disease infection that penetrates the immune system and not only causes the disease mentioned previously, but also causes complications or disease conditions that are very dangerous to people's lives and may be the cause of death for nearly 100% of people, and the main cause of this disease is fungi. Aspergillus fumigatus [17] Therefore, it has become one of the foundations of research and investigation to find more appropriate and effective methods for accuracy in diagnosis .They are available at affordable prices to diagnose fungal infections and initiate the necessary treatments to reduce the speed at which these deadly fungi spread [20] Molecular markers of various types are known at a very advanced level and are used at various levels in scientific research [1] Assessing genetic diversity is an essential use of molecular markers [1 - 4] Thus, RFLP [5] RAPD [6] AFLP [7] SSR (5,8), ISSR [9] and many other marker systems. Which relied on the use of DNA to study mutations, genes, new sequences, etc. (10). Due to their high polymorphism, co-dominance, and partial reproducibility, ISSR markers have been described as promising DNA markers in a variety of applications. Techniques include the use of ISSR-PCR methods, secondary DNA markers, and DNA profiling. Identify the types of fungi. [11] This technique has been used to produce multiple classes of related DNA polymorphisms [9] and as a result, ISSR markers have proven to be very effective in estimating genetic diversity in various studies [12] [13] The most accurate measurement method available in our work is by amplifying specific regions between effective repeats in opposite faults and the non-amplifiable offset between two identical small satellite regions (ISSR) [16]. Many technical research studies have been done on these types and correct types [14] We have contacted our private messages and their development in signs [15] These remarkable properties have led to the discovery that ISSR markers are relatively easy to develop and highly used in applications such as genetic mapping, phylogenetic analysis, population structure analysis, diversity/line identification, and fingerprinting. Microsatellite sequences are used as primers in a polymerase chain reaction (PCR) that generates multilogue markers through polymorphic simple sequence repeat (ISSR) technology. All data generated using the ISSR technique are useful in fields. Genetic diversity allows for quick conservation decisions to be made without taking environmental data into account [4] The main advantage of

ISSR is that primer synthesis needs no sequence data. Very little template DNA is required because these methods involve PCR. Moreover, ISSRs exist at random throughout the genome. As a result of acquired multilogue imprinting patterns, can be applied to investigations dealing with genetic personality, fathering, cloning purposes lineage identification, and taxonomic evaluations of closely related species. It is also believed that ISSRs are useful in genetic mapping studies. A disadvantage of ISSR as a multifocal modality is that similarity among segments of similar size can sometimes be reached.

2. Material and methods

1.2. Culture media

Ready to use powdered media Medium Company Origin Saburou Dextrose Agar medium (SDA) Hi media India, Potato Dextrose Agar medium (PDA) HI media India, Sabourin Dextrose Broth medium (SDB) HI media India. The medium was prepared according to the instructions fixed on their containers as indicated by the manufacturer. After adjustment of pH, they were sterilized by autoclaving at 121ºC for 15 min., B/PDA medium contained, Potato infusion(200g), Dextrose(20g), Agar(15g), and Distal water(1000ml). Prepared by dissolving these compounds in D.W. and the pH was adjusted to 6.3 and then sterilized by autoclaving (Pitt and Hocking.,. A stock solution of chloramphenicol (10µg/ml) To prepare it, 100 mg of chloramphenicol was immersed in 10 milliliters of D.W. (AL-Ajnabi, 1999).
6-2. Lactophenol cotton blue stain, A/Phenol (0.05g), Glycerol(40ml), Latic acid(20ml), D.W (20ml)
The DNA fragment was separated into different Concentrations. It was used as the following):
(0.5%) was used for extracting DNA samples then add (1%) was used for visual checking of RAPD products with (1.5%) was used for ISSR products. It was made by dissolving a single gram of ethidium bromide in hundred to one hundred milliliters of sterile D.W. and storing the bottle in the dark. Because ethidium is a potent mutagen and a highly carcinogenic material, it must be handled carefully during the weighing process. During handling, made from ethidium bromide intercalates between the bases of DNA and fluoresces when exposed to ultraviolet (UV) rays. The pre-mix contents

Components	Reaction size (20µl reaction)
Taq® DNA polymerase	1U
dNTP, $_{CTP}$, dGTP, dTTP	250µM
Tris-HCl (pH 9)	10Mm
KCl	30Mm
MgCl 2	1.5Mm

2.2. ISSR: primers

We use ISSR primers. Their synthesis was done by Pioneer Korea. The final concentration of 10 p/mol was attained by lyophilizing the material that was dissolved in sterile D.W. Table 1 lists the primers along their corresponding patterns.

Table 1: Sequence of ISSR-PCR Primers

No.	Primers Name		Sequence (´5-´3)
	$ISSR_3$		AGTGAGTGAGTGAGTG
2.	$ISSR_4$		GACACGACACGACACGACAC
3.	$ISSR_5$		AGAGAGAGAGAGAGAGAGAG
4.	$ISSR_9$		CTCTCTCTCTCTCTCTCTG
Materials		Last-minute focus	One tube's volume
PCR premix		1x	5µI
Deionized D. W		—	17 µI
Primers		10 p/mol	2µI
DNA the template		100 ng	1µI

2.3. ISSR –PCR

It requires 40 cycles total between that initial denaturation and final extension. The ISSR program will appear in the following table:

Actions	The value of the temperature	Moment (minutes)
Initial denaturation	94ºc	5 min
Denaturation	94ºc	1min
Annealing	50ºc	1min
Extension	72ºc	1min
Final Extension	72ºc	10 min

Around 12µI of PCR amplified products were separated by electricity in 1.5% agarose gel (2hrs, 7V/cm, 1xTris-borate buffer). Gel was stained with the ethidium bromide; PCR products were pictured by a UV transilluminator and then were imaged by gel documentation in the system. The amplified products' ISSR-PCR items were estimated by comparing them with the marker dye mixture and performing this process.

2.4. Finding the discriminatory power and primer efficiency

Each primer's efficiency discriminatory power was determined using a certain formula: Efficiency correlates Discriminating effectiveness terms of how many bands to the same primer is calculated as the output number of bands to each primer compared to the total amount of unique bands to all primers. X100 Primer efficiency was measured as the number of polymorphic bands multiplied by the overall number of bands in each primer. The result ranged from 0 to 1. The proportion of polymorphic bands existing in each primer in comparison with the total number of polymorphic bands in all primers used to determine each primer's discrimination power (Younan et al., 2010).

3. Results and discussion

3.1. Being alone and verification of a. fumigatus via different sources

Out of a total of ten specimens from different sources, 7 isolates were used for the removal of DNA (table 4) (5 environmental samples and 5 scientific backing samples). The clinical isolates were examined using direct 10% KOH investigation, which shows the development of conidial heads in bracelets basipetal from phialides, as well as septet hyphae and dichotomous branching. Conidia are transported directly on widely clavate vesicles in the absence of medullae. Employing an alkaline potassium hydroxide, as the solution. Because of this, the fungus in the pieces under examination was unaffected and naturally distinguished from different materials which could be mistaken for the fungus.

Table 2: Isolates of A. Fumigatus from Various Sources

Sample No.	Samples	Sources
AFU1	Sputum aspergillosis	Clinical
AFU2	A sample of river water	Surroundings
AFU3	Hospital to earn contamination (Swap)	Clinical
AFU4	soil-round plant roots	Surroundings
AFU5	Zea mays grain	Surroundings
AFU6	External Otitis (Swap)	Clinical
AFU7	A sample of air	Surroundings

3.2. Molecular examination

3.3. DNA extracted from isolates of a. fumigatus: concentration and purity

A straightforward and genetic DNA extraction protocol was used to extract all of the genomic DNA from the fungal isolate samples using a commercial kit (Pioneer-Corporation). The purity of the isolated DNA ranged between (1.6 and 1.8), and the yield of the DNA obtained from fungal growth was in the range of (470-580) µg/ml. The standard method is used to measure concentration and purity (Sambrook et al., 1989).

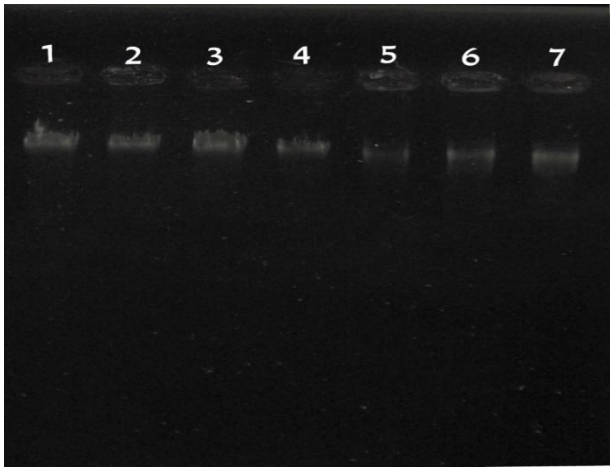

Fig. 1: Total Genomic DNA from Specimens Was Extracted Using A Commercial Kit And Electrophoresed on An Agarose Gel (Bioneers).

3.4. Results of using the ISSR indicator

Using the ISSR3 primer, the genomic DNA of the fumigatus isolates was amplified; the PCR results showed genetic diversity for the isolates. The overall number of bands [25] which was divided into 6 primary bands with sizes varying from 350 to 1200 bp, is shown in (figure -4). The six bands, which varied in molecular weight for spans of 350, 400, 700, 800, 900, and 1200 bp, were polymorphic. There were no distinct or monomorphic bands created by the primer. The variation of the isolate numbers {1, 2, 4} in two groups, {3, 6, 7} in five bands like and {5} in four bands. (table -5) provides a summary of all the data collected in (figure 4). among individuals After having been amplified, the genomic DNA of 7 A. fumigatus isolated strains was revealed utilizing the ISSR4 primer to assess their genetic diversity. This was clarified in (Table 5), which in turn generated a total of 19 bands, each with a size ranging from 250 to 1000 bp and divided into 7 major bands. These seven bands consisted of seven polymorphic main bands with molecular weights of 300, 400, 600, and 1000 bp, as well as the three separate bands for isolate numbers 1 and 7 with molecular weights of 250, 350, and 500 bp each. Table (6) summarizes all information obtained from (figure 4). These included the amplified binds' molecular weights and the possibility that they had bands. Amplification products with go [3] band of isolate amount {1, 5, 6} and (4) ensembles of isolate number {2, 4} were obtained from this primer. and [2] isolate bands with a number {7}. The outcomes of on the agarose gel, the PCR reaction of primer ISSR5, which reacted with the genomic DNA of the A. fumigatus isolates, displayed up as 26 bands overall for all isolates. Their distribution was made up of six main bands ranging in size from 250 to 1000 bp, and six polymorphic bands with molecular weights between 250, 350, 450, 550, 650, and 1000 bp. Bands included the {2} band produced by isolate number {1} to [3] bands produced by isolate numbers {6, 7}, but [4] bands had been generated by isolate numbers {2} and {4}. And separate {3, 5} to create a [5] band. All of the information from (figure 5) has been collected in (table -6). Between these were the amplified bands' molecular weights, whether they existed or inertia, and included were the distinct bands, their inclusion or absence, and the atomic weights of the amplified bands.

By transforming their genomic DNA, the ISSR9 primer PCR results displayed genetic diversity amongst A. fumigatus isolates. Twenty main ensembles total for all isolates were visible on an agarose gel as a result of a primer ISSR9 PCR reaction that behaved with genomic

DNA from each of the A. fumigatus isolates. Ten major bands, ranging in size from 200 to 1400 bp, consisted of them. Ten of these bands had polymorphic traits; their molecular weights varied from 400 to 1400 bp. For isolate plenty {2, 3}, (Table-6) showed five different bands of sizes that vary ranging from 200 to 900 base pairs (bp). The number of bands created by the mentioned primer had these results reveal that the ISSR primer is distinctive in knowing elements that set each isolate out from the others. Contrary to the random amplification technique, which is less skilled in differentiating isolates and identifying bands, it can be depended upon in the removal and examination of Aspergillus isolates, whether these are environmental or medical isolates. Particular to specific molecular weight for a specific kind of isolate, the first analysis in a (table -5). Showed that the use of an ISSR primer of ISSR4 is an excellent primer in connection with the primary medical isolation of those infected with aspergillosis. It is sufficient to find the first band in the molecular weight of a specific band in the weight of the other three. 500bp and 350bp compared to finding products specialized in environmental insulation isolated from leaving the dishes open to the air, as it was able to bond and separate a specific unit package with a molecular weight of 250bp. It proved an inability to discern between the isolate at a specific weight and a different isolate once compared to using the third ISSR4 primer. On reviewing) (table -6), it appears that ISSR-9 primer executes very well in the diagnosis of environmental isolate number two. This isolate was obtained from river water and contained a particular kind of Aspergillus The efficiency rate of ISSR primer number nine to create four packages within the molecular weight range of (900, 600, and 350) bp was excellent. 250bp molecular weight, in addition to the packet Furthermore, he was successful in distinguishing across one isolate for the medical isolate the fact that was taken from hospital patient beds and covered with a molecular weight of 200 bp. Comparison with its classmate, ISSR5, which was less reliable than its corresponding counterpart, Primer ISSR 9, in this group of isolates despite practicing distinct bands at varied molecular weights within the isolates.

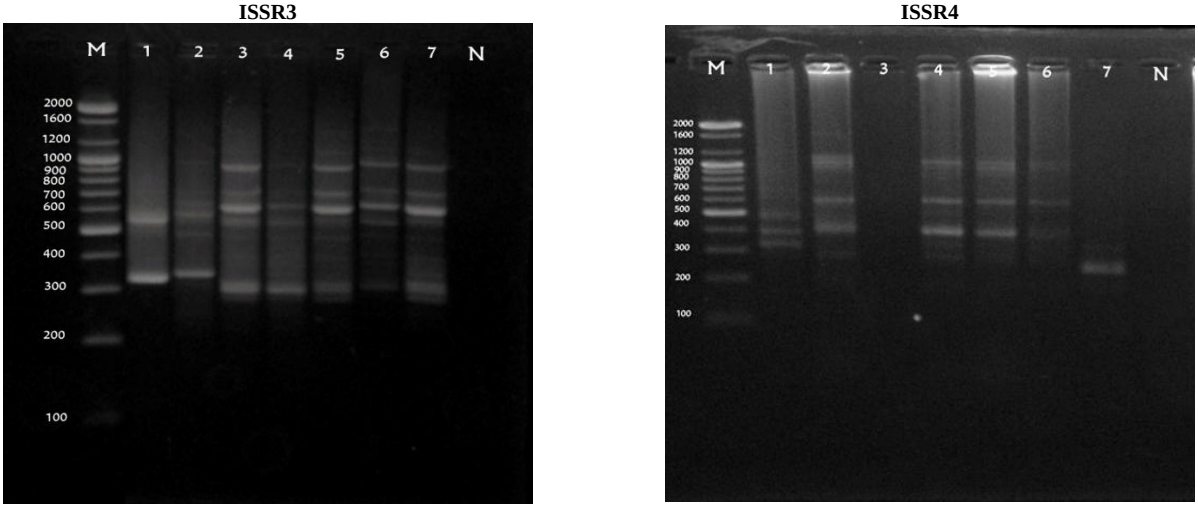

Fig. 2: Ethidium Bromide-Based 1.5% Agarose Gel Electrophoresis of the PCR Product Utilizing the ISSR Primer Is Depicted in. M: A 100 Bp DNA Ladder.

Table 3: The Polymorphic Band (White) with Their Molecular Weight for ISSR3 and ISSR4 Primers

NO.	The molecular weight of packets	1	2	3	4	5	6	7
1	1200	0	0	1	0	1	1	1
2	900	0	0	1	0	1	1	1
3	800	0	0	1	0	1	1	1
4	700	1	1	1	1	0	1	1
5	400	1	1	0	0	0	0	0
6	350	0	0	1	1	1	1	1
NO.	The molecular weight of packets	1	2	3	4	5	6	7
1	1000	0	1	0	1	1	1	0
2	600	0	1	0	1	1	1	0
3	500	1	0	0	0	0	0	0
4	400	1	1	1	1	1	1	0
5	350	1	0	0	0	0	0	0
6	300	0	1	0	1	0	0	1
7	250	0	0	0	0	0	0	1

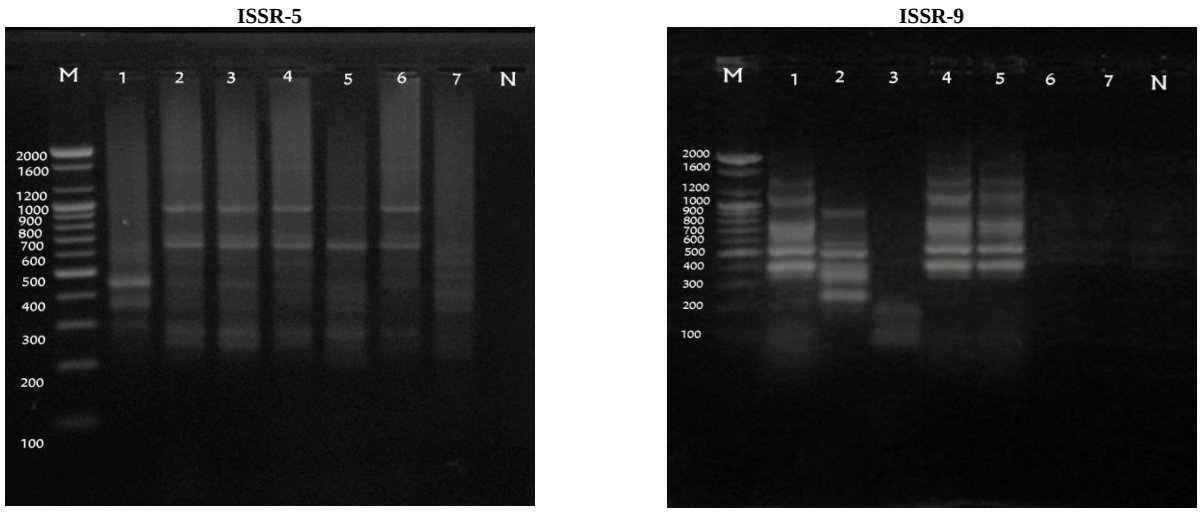

Fig. 3: Ethidium Bromide-Based 1.5% Agarose Gel Electrophoresis for the PCR Product Using ISSR Primer M: DNA Ladder with 100 Bp. N Stands for Negative Control. It1, It2, It3, It4, It5, It6, and It7 form the Lanes.

Table 4: The Polymorphic Band (White) with Their Molecular Weight for ISSR5 and 9 Primers

NO.	The molecular weight of packets	1	2	3	4	5	6	7
1	1000	0	1	1	1	1	1	0
2	650	0	1	1	1	1	1	0
3	550	0	1	1	1	0	0	1
4	450	1	0	1	0	1	0	1
5	350	1	0	0	0	1	0	1
6	250	0	1	1	1	1	1	0
NO.	The molecular weight of packets	1	2	3	4	5	6	7
1	1400	1	0	0	1	1	0	0
2	1100	1	0	0	1	1	0	0
3	900	0	1	0	0	0	0	0
4	750	1	0	0	1	1	0	0
5	600	0	1	0	0	0	0	0
6	500	1	1	0	1	1	0	0
7	400	1	0	0	1	1	0	0
8	350	0	1	0	0	0	0	0
9	250	0	1	0	0	0	0	0
10	200	0	0	1	0	0	0	0

4. Genetic distance

The seven A. fumigatus isolates had a genetic similarity ratio ranging from (0.10253-0.97826), as shown in (table-8). The isolates with amounts {1} and {2} possessed the highest similarities (0.97826) at 97%. The isolate numbers {5} and {7} had the lowest degree of similarity (0.10253), 10%. All introduced isolates behaved as predicted: the first group comprised two clusters, a first of and that contained isolates {5} and {7} with a lower genetic distance (0.1025) Ten percent of these were isolates {5} and {7}, which began from grain samples and related to the same species, and the rest were derived from air samples; isolate {1}, on the other hand, originated from an Aspergillosis patient and created a distinct subgroup within the main group. In the present study, the second main group was made up of two sub-clusters, the first cluster contained isolate numbers {3,6,4}, and this clustering was divided into subgroups, first the subgroup contained isolate number {3} and {6} with genetic distance (0.21556), 21% these were isolates found number {6} which came from patients with Otitis externa source and isolate number {3} came formed hospital contaminations, because isolate amount {3} may develop pathogen, while isolate number {4} come from the soil a rounded plant roots source and of the same group and isolate number {2} came in the river water sample and formed separate sub-cluster from isolated organisms number {3} and {6} in the same main group, the high ratio in the table (8) between isolate number {1} or {2} was because isolate number {1} came from individuals sample but isolate number {2} came from a sample of river water. Several investigations have shown that ISSR-PCR and RAPD processes are similar in that they both depend on sensitive reaction circumstances. To achieve the highest levels of specificity and product yield, PCR situations must be tuned (Williams et al., 1990; Hashimi, 2009). To achieve a successful ISSR-PCR reaction, optimization of the PCR conditions—including temperature, number of cycles, reagent, and other parameters—is crucial (Omar, 2009). This highly reproducible approach, which uses primers with microsatellite DNA sequences and degenerate anchored at the 5 ends, can detect both intraspecific and interspecific DNA polymorphisms and applies to a broad range of fungal species Haunt et al. (1996). Because the evolution rate within ISSR is substantially greater than that of most other types of DNA, the hypothesis was supported by ISSR-PCR in contrast to most other techniques, including RAPD-PCR. The analysis of ISSR-PCR reaction for seven A. fumigates isolates found the absence or involvement of bands, differences in molecular weight, and degree of amplified bands (Mayer et al., 2000). the research of Kernodle et al. (1993), various factors, involving primer structure and annealing sites in the genome, can influence the variance within the assortment of bands boosted using various primers. In the current study, nine different structure Primers were used to examine the genetic diversity within seven A. fumigates isolates, and the findings showed an enormous amount of variation. However, since the concentration of the genetic material was not controlled, the band intensity was not taken into consideration as well. Compared to other molecular techniques, the minimal sequence repeat (ISSR) technique can produce more polymorphisms and triumph over all the limitations of various methods in determining genetic diversity along with genetic distances between them. To determine whether medicinal and environmental issues Aspergillus species can be divided into distinct genetic groups, ISSR markers were assessed in this study. The aims were to: characterize the genetic diversity of seven groups, categorize them, and contrast the molecular marker results with the morphological data-derived results. Simple sequence repeat (ISSR) constitutes a handful

of the effective genetic markers. High polymorphism ISSR markers are useful for research on genetic diversity, markers for genome mapping, and

Table 5: The Study's Unique ISSR Primer Characteristics Included the Primer's Name, Total Number of Bands, Polymorphic Band Determine, Unique Band Measure, Polymorphism Percentage, Primer Efficiency, and Discrimination Value

NO.	Name of primers	Total number main bands	Number of unique bands	Number of polymorphic bands	Polymorphism %	Primer efficiency	Discrimination Power%
1	ISSR3	6	0	25	100	1	13.3
2	ISSR4	6	3	16	84.2	0.84	8.55
3	ISSR5	10	0	15	27	0	8.02
4	ISSR9	6	5	26	100	1	13.9

Table 6: Genetic Distance Values Between Isolates of A. Fumigatus Measured Corresponded with Nei and Lei (1979)

	1	2	3	4	5	6	7
1	0.0000						
2	0.9782	0.0000					
3	0.8155	0.6824	0.0000				
4	0.7156	0.4507	0.7055	0.0000			
5	0.5418	0.9455	0.9485	0.8674	0.0000		
6	0.7803	0.7418	0.2155	0.2155	0.8030	0.0000	
7	0.55263	0.84184	0.98682	0.87868	0.10253	0.76330	0.0000

5. Examining genetic distance

A. fumigates isolates' genetic distances were computed using the multivariate analyses System Version 1.80 put and taxonomy, which depend on the standard. Bands among all types; an increase in band number results in a decrease in genetic distance, and vice versa. The A. fumigate isolates under investigation have their genetic distance values communicated in the (tables 8). The genetic separation between the seven A. fumigatus isolates had been identified by looking at the intersection of every two isolates. The greatest genetic distance, 0.97826, was found between isolates (1)and (2). The lowest value between isolates (5)and (7)is 0.10253. then Examining the company's operations, the dendrogram was created using. The Nei and Lei's (1979). Quantification of gene distance using UPGMA group analysis (Figure 6) showed two noteworthy clusters and show gene associations among the seven isolates of A. fumigatus. • The second main group was divided into two subsections: specimens (3, 6, and 4)had been placed in the initially formed subcluster, and isolate No. (2)was contained in the secondary subcluster. The first major group consisted of two sub-clusters: isolates (5)and (7)made up the first sub-cluster, and isolates No. 1 made up the supplementary sub-cluster.

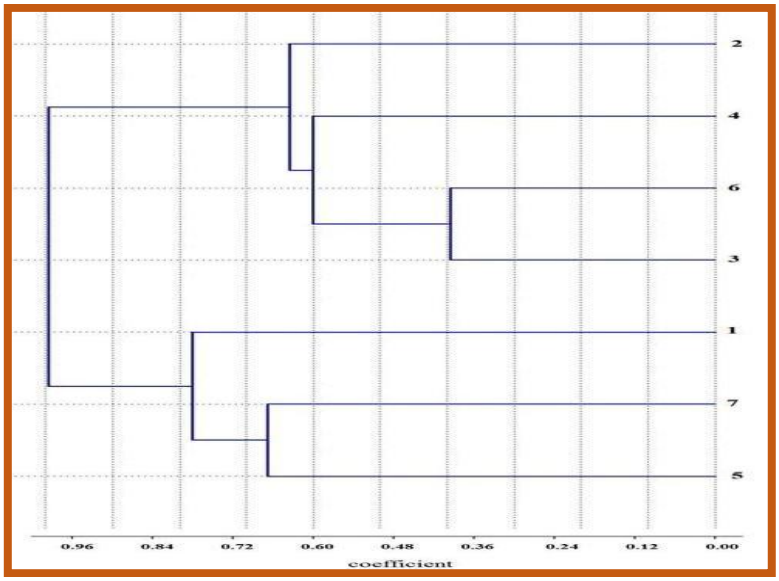

Fig. 4: Utilizing ISSR Data, A Dendrogram Displaying the Genetic Fingerprint and Relationships between A. Fumigatus Isolates Was Generated.

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