Identification of *Cladosporium* sp. Fungi by *in- silico* RFLP-PCR

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Abstract:

Cladosporium sp. plays an important role in human health, it is one of the pathogenic fungi which cause allergy and asthma and most frequently isolated from airborne spores. In this study, a couple of universal PCR primers were designed to identify the pathogenic fungi *Cladosporium sp*. according to conserved region 5.8S, 18S and 28S subunit ribosomal RNA gene in *Cladosporium* species. *In silico* RFLP-PCR were used to identify twenty-four *Cladosporium* strains. The results showed that the universal primer has the specificity to amplify the conserved region in 24 species as a band in virtual agarose gel. They also showed that the RFLP method is able to identify three *Cladosporium* species by specific and unique restriction enzymes for each one. These species are *Cl. halotorenas* by the two unique enzymes *BsaXI* and *MobII*, the other species is *Cl. colrandse* by two enzymes *BccI* and *BtsCI*, while the third species is *Cl. aciculare* by one enzyme *BceAI*. Each enzyme forms two bands in virtual agarose gel as a results of cutting the DNA by the enzyme, where the rest twenty – two species share more than one restriction enzymes. This method is active and rapid for identifying *Cladosporium* genus and three species by computational bases methods before applying it in the lab for more accuracy, efficiency, and specificity of designed primer to get good results in a short time.

Key words: Bioinformatics, *Cladosporium sp*, *In silico* PCR, Restriction enzymes.

Introduction:

Using the morphological methods for identifying fungal species needs a very long experience and skills to differentiate between a very wide range of fungal species. Molecular methods are more accurate from those classical methods especially when using specific primer for each species and genes (1).

The PCR techniques have been widely used in mycological study, the success of polymerase chain reaction depends on more than one factor; the most important and critical one is the ability of the primer to amplify the specific DNA template. To check this, the researcher needs to make the PCR reaction with all regents. In case the reaction failed to form a product in agarose gel, the reason behind this among more than one factor is needed to be known. The amplified DNA by using Thermocycler (PCR) is a basic tool in molecular biology and the most common technique used in laboratory researches and DNA studies.

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The principle of this tool has been widely used and applied out of many other simple or complex nucleic acid amplification technologies (NAAT) beside laboratory which deals with experiments for amplification of nucleic acid technologies, *in silico* or virtual (bioinformatics) PCR have been developed, along within in-*silico* PCR analysis. *In silico* NAAT analysis is a very useful and efficient complementary method to ensure the specificity and efficient of primers or probes, (2).

For a long time, the internal transcript spacer (ITS) of the DNA has been used as a region for analyzing fungal diversity in many environmental sites, and has recently been selected as a biomarker for fungal DNA diagnosis, the ITS region has the ITS1 and ITS2 sequences separated by the 5.8s gene, and it is position between 18s (SSU) and 28s(LSU) genes, which is represented nrDNA repeated unit. Conserved regions in DNA or protein amino sequences are a good candidate for functional elements and thus conserved genes and sequences are similar or the same sequences in nucleic acids (DNA and RNA) or proteins among species or within a genome. Conserved regions indicated that sequences have been maintained by natural selection, a highly conserved sequence

strongly remained relatively unchanged for keeping the phenotypic tree. Across many generations, nucleic acid sequences in the genome of an evolutionary steps are gradually changed over time due to the random changed like mutations and deletions and recombining of many sequences or may delete some of them as a results of chromosomes rearrangements. Conserved regions are sequences which resist any forces to change their sequences like mutation and another effect to conserve their position in the genome, (2).

Ribosomal RNA specification is very important in the development and transformation, thus taxonomy and medicine. rRNA is one of only a few gene products present in all cells; for this reasons, a gene that encodes the rRNA (rDNA) is studied to identify an organism's classification group, estimating related and rates of species differences. Consequently, many thousands of rRNA sequences are known and stored in specialized databases NCBI (National Center for Biotechnology Information). rRNA is a target of numerous clinically relevant antibiotics and shown to be the most important tool in taxonomy and identification.

The *Cladosporium* is a very interested fungus in the environments as they are distributed in a wide level outdoor and indoor, (3). The *in silico* PCR is the best method to test the efficiency of the designed primers and many other reactions, in this study the *in silico* PCR was used to test the designed primer and to find and compare the specific restriction enzyme among twenty-four species of *Cladosporium* to used them as a marker for identification.

Materials and Methods:

The rRNA ribosomal subunit of twenty-five *Cladosporium* strains sequences was obtained from NCBI web site, Table (1).

Table 1. *Cladosporium* Species with their identification numbers.

Strain No. Accession No	Species
1. *Cladosporium sphaerospermum*	MF473267.1
2. *Cladosporium halotolerans*	MF473072.1
3. *Cladosporium cladosporioides*	EF405864.1
4. *Cladosporium wyomingense*	MF473315.1
5. *Cladosporium parasubtilissimum*	MF473170.1
6. *Cladosporium needhamense*	MF473142.1
7. *Cladosporium coloradense*	MF472945.1
8. *Cladosporium herbarum*	DQ093757.1
9. *Cladosporium xanthochromaticum*	MF473322.1
10. *Cladosporium vicinum*	MF473312.1
11. *Cladosporium tenuissimum*	MF473305.1
12. *Cladosporium subcinereum*	MH161229.1
13. *Cladosporium allicinum*	NR_152266.1
14. *Cladosporium silenes*	NR_111270.1
15. *Cladosporium herbaroides*	NR_119655.1
16. *Cladosporium chalastosporoides*	NR_119838.1
17. *Cladosporium phlei*	NR_120013.1
18. *Cladosporium acalyphae*	NR_119835.1
19 *Cladosporium antarcticum*	NR_121332.1
20. *Cladosporium tenellum*	NR_119662.1
21. *Cladosporium versiforme*	NR_152297.1
22. *Cladosporium penidielloides*	NR_152295.1
23. *Cladosporium aciculare*	NR_152294.1
24. *Cladosporium ipereniae*	NR_152290.1
25. *Cladosporium arthropodii*	NR_120011.1

Universal primer design for detecting *Cladosporium* species:

A set of primer was designed depending on conserved regions of twenty-five of *Cadosporium sp.* sequences by using Clustal W tools to assess the fungal conserved regions, (4).

A set of a primer depending on conserved regions of ribosomal RNA subunit (18S, 5.8S, and 28S) of twenty-five *Cladosporium sp.* was designed by using the Geneious tool, (version 10.1). This program is able to diagnose, detect and compare the similarity region in relative species, the primer design is a very critical step to any successful PCR amplification. The designed primers specificity and sufficiently to amplification for all *Cladosporium sp.* studded species was tested by using NCBI primer blast, the DNA amplified by the designed primers was tested by using virtual agarose gel electrophoresis and the size of bands formed by amplification was measured, (5).

The restriction fragment length polymorphisms (RFLP):

The RFLP is a technique that can divide the DNA in-to more than two fragments in special sites according to the type of the restriction enzymes and sequences. The DNA, which is cut to different size segments, can be detected by using agarose gel electrophoresis. All the amplified DNA of *Cladosporium* sp. by universal primers were subjected to the restriction enzymes with the help of NEBcutter tool, (6). The enzymes which digested and cut the DNA to segments and unique for specific *Cladosporium* sp. have been chosen.

Results and Discussion:
Universal primer efficiency test:

According to twenty-five *Cladosporium. sp.*-ribosomal RNA (rRNA) conserved region; two sets of universal primer were designed using *in silico* programs; Geneious tools, (v. 11.1.5), (Fig. 1 and 2) and tested by SnapGene and Amplifix tools, (Table 1) (7). The specific primer is able to amplify twenty-four species out of twenty- five.

Table 2. Universal primer used for Amplification of *Cladosporium* species

Primer set	Sequences	Length bp	GC%	Temperature ° C
Forward	TTGTCCGACTCTGTTGCCTC	20	55	56
Reverse	TTCCACAACGCTTAGGGGAC	20	55	56

The band size of the amplified region of all species was 363 bp; (Fig. 3), the species *Cl. penidielloides* have not been detected by the designed primer and this may be due to the short fungal sequences in NCBI. The sequences are shorter than the other species which made the primer out of its beginning and end sequences. It's not possible to make the primer start point compatible with the shorter sequences; this can make the amplified band size very small in agarose gel, (8).

Figure 1. Forward primer
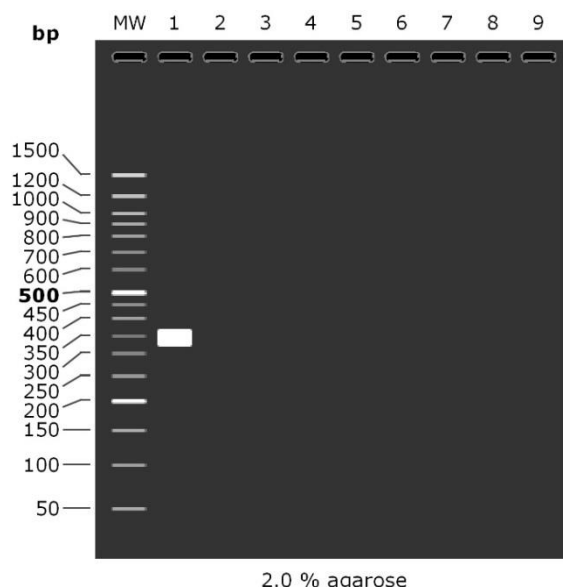

Figure 2. Reverse primer

Figure 3. Amplified DNA by using universal primer for *Cladosporium sp*. in agarose gel showed the single band in size 363 bp

Identification of *Cladosporium* species by RFLP

The results of using RFLP technique to identify *Cladosporium* sp. showed that there are three species of *Cladosporium* fungi among the 24 amplified DNA species that can be identified by using unique restriction enzymes for each species and not sharing them with other species. The remaining species are shared with one or more RE and can't be used for identification, while the RE *ApekI* and *AseI* were found in all *Cladosporium* species and can be used for the differentiation between *Cladosporium* genus and other fungi genus like *Aspergillus* in which these enzymes have no sites in seven *Aspergillus* species sequences, (Table 3). The species *Cl. aciculare* have a one unique RE; *BceAI* ,(Fig. 4), cutting the DNA into two segments in sizes 310 and 54 bp, Fig. (5).

Table 3. *Cladosporium* species and unique RE enzymes

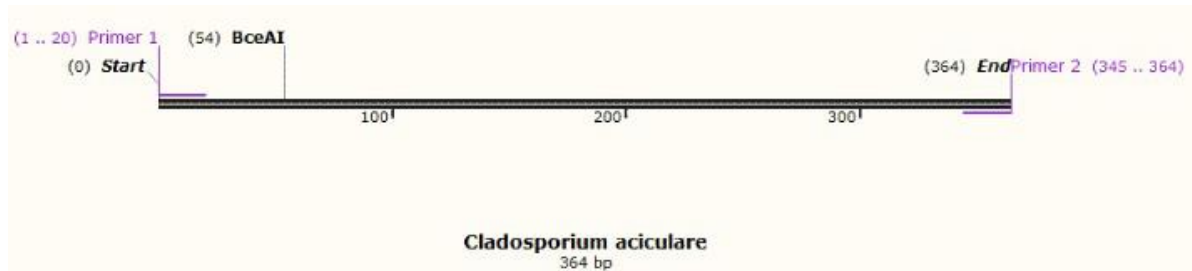| Unique Enzymes in sp.	Enzyme	Specificity	products
Cl. aciculare	*BceAI*	ACGGC(N)12 NN	54/56
Cl. halotorenas	*BsaXI*	N (N)9AC(N)5CTCC(N)7 N	18/15+48/45
	MboII	GAAGA(N)7 N	163/162
	BccI	CCATCNNNN N	47/48
Cl. colrandse	*BtsCI*	GGATG NN	58/56
	FoKI	GGATG(N)9 NNNN	65/69
All 24 *Cladosporim* Sp.	*ApekI* *AseI*	Not found in *Aspergillus* species: A. fumigatus, A. niger, A. neoafricans A. flavus, A. nidulans A. fischeri, A. novofumigatus	

Figure 4. Restriction enzyme site in amplified DNA of *Cldosporium aciculare*
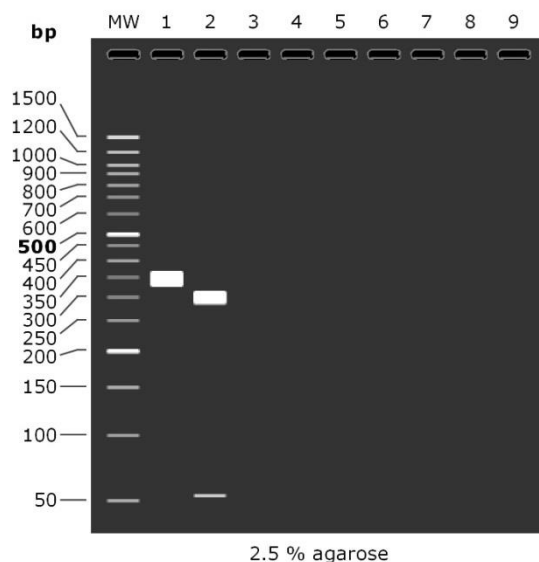
Figure 5. 2% of an agarose gel of *Cl. aciculare* fungi by Geneious tool: MW: molecular weight 1. DNA product of fungi 2. Cutting the DNA by RE

While the species *Cl. halotorenas* have two unique RE; *BsaXI* in two sites and *MboII* in one site in the amplified DNA, (Fig. 6).
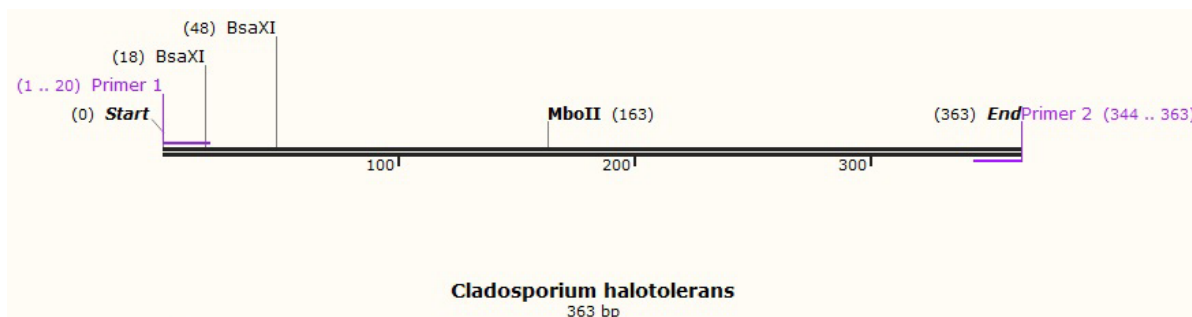

Figure 6. Restriction enzymes site in amplified DNA of *Cldosporium halotorenas*

The virtual agarose gel electrophoresis showed the cutting segments, one by *BsaXI* which forms two segments one in size 315 bp and the other in size 48 bp, while the second RE forms two segments in size 200 and 163 bp respectively, (Fig. 7).
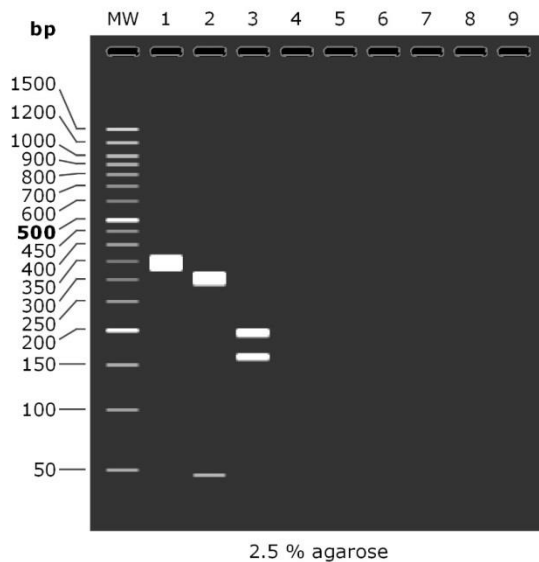

Figure 7. 2.5 % agarose gel : MW: Molecular weight, 1. Fungal *Cl. halotorenas* DNA amplified, 2. Two DNA segments by *BsaXI* enzyme, 3. Two DNA segments by *MboII* enzyme

And the species *Cl. colrandse* have three unique RE; *BccI* , *BtsCI* and *FoKI* (Fig. 8). The first one forms two segments in size 47 and 316 bp,

the second enzyme forms segments in size 58 and 305 bp and the last one forms segments in size 65 and 298 bp (Fig. 9).
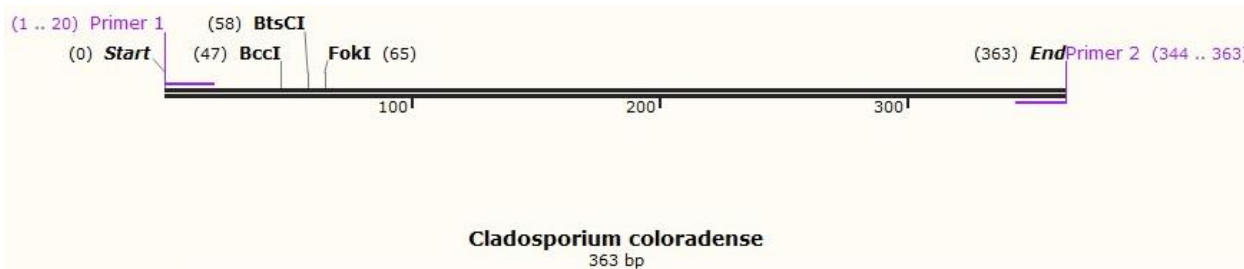

Figure 8. Restriction enzymes site in amplified DNA of *Cldosporium colorandse*
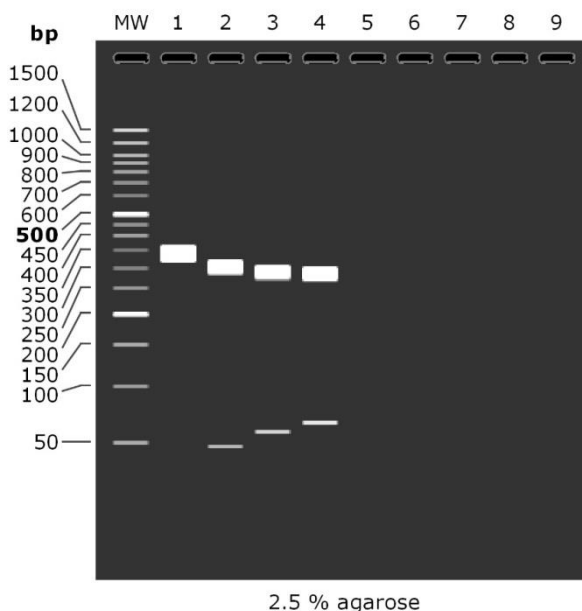

Figure 9. 2.5 % agarose gel: MW: Molecular weight, 1. Fungal *Cl. colrandse* DNA amplified, 2. two DNA segments by *BccI* enzyme, 3. Two DNA segments by *BtsCI* 3. Two DNA segments by enzyme *FoKI*

The results showed the possibility of using RFLP technique for identifying the fungi species which have a variation in sequences and far away in phylogenetic tree, as shown in Fig. 10. The very close species have shearing most restriction enzymes and cannot be used as a tool in identification methods; but with those are far away in genetic. The species which have a unique restriction enzyme have differences in their genetic characteristics, (9).
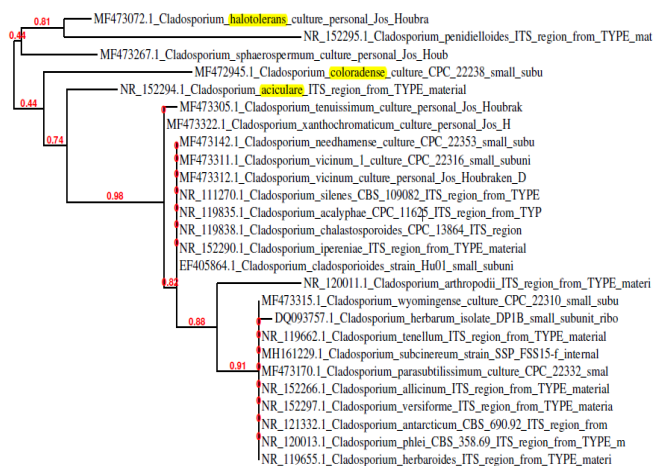

Figure 10. Phylogenetic tree of all *Cladosporuim* specie

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تشخيص انواع الفطر *.Cladosporium sp* بواسطة تغاير قطع التقييد لتفاعل البلمرة المتسلسل (RFLP) المحوسب

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الخلاصة:

يلعب الفطر *Cladosporium* دورا مهما في صحة الانسان فهو احد الفطريات المسببة لامراض الحساسية والربو ومن اكثر الابواغ المحموله في الهواء ، ولاهمية هذه الفطر شملت هذه الدراسة تصميم بادئ عام لتشخيص 25 نوعا للفطر *Cladosporium* طبقا للمناطق المحفوظة في تسلسل القواعد النتروجينية في مواقع وحدات الرايبوسوم 5.8S و 18S و 28S في هذه الانواع. استخدمت طريقة تغاير قطع التقييد لتفاعل البلمرة المتسلسل (RFLP) لتشخيص 24 نوعا من انواع الفطر التي تم تشخيصها باستخدام البادئ العام . اجريت جميع التجارب باستخدام برامج البايولوجي الجزيئي على جهاز الحاسوب والتي اظهرت نتائجها فعالية البادئ المصمم في تشخيص 24 نوعا من اصل 25 نوع وايضا امكانية تشخيص والتمييز لثلاثة انواع من الفطريات باستخدام الانزيمات القاطعة المتخصصة على مستوى النوع وهي *Cl. halotorenas* باستخدام ثلاثة انزيمات متخصصة و *Cl. colrandse* باستخدام انزيمين و *Cl. aciculare* بانزيم واحد. توفر هذه التقنية طريقة فعالة في فحص مدى كفاءة البادئات المصممة والمختارة لاجراء تجارب الـ PCR افتراضيا قبل استخدامها في المختبر وكذلك استخدام طريقة RFLP عن طريق ايجاد الانزيمات القاطعة الخاصة على مستوى النوع للاجناس المهمة صحيا لاستخدامها في المختبر لاختصار الوقت والجهد ونجاح عمليات تضخيم وتفاعل انزيم البلمرة PCR .

الكلمات المفتاحية: الفطر *Cladosporium sp.* ، علم الاحياء الحاسوبي، مضاعفة الحامض النووي المحوسب، الانزيمات القاطعة.