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Genetic Diversity of Some *Trichophyton* Species Isolated from Patients with Cutaneous Mycoses

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بسم الله الرحمن الرحيم

وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي
وَمَا أُوتِيتُم مِّن الْعِلْمِ إِلاَّ قَلِيلاً

صدق الله العلي العظيم

سورة الإسراء
الآية (85)

Dedication

To the candle of my life , strength, confidence and the person who made me gain success and happiness…

my father

To the love , warmness, life smiles…

my mother

To the refuge in my life …

My family

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Summary

A total of 150 specimen (patient) were obtained from individuals of all ages and both sexes who were diagnostic clinic by dermatologist to have a cutaneous fungal infection, including scrapings of the skin (n = 100) and nails (n = 50), The Patients were visited to dermatology outpatient clinic of Marjan teaching hospital , and dermatology outpatient clinic Al‑Sadeq teaching hospital , these specimens diagnostic clinically by dermatologists between April and October 2022 . Then the specimens were cultured in selective media and identified by biochemical tests and using specific primer for *Trichophyton mentagrophytes* ,*Trichophytin rubrum and Trichophyton violaceum* .

From total of (150) specimen , only 50 specimen of *Trichophyton species* isolated from skin and nail scraping were detected by direct microscope examination (10 % KOH) , culture on sabouraud dextrose agar or other media and biochemical test .And Confirmed diagnosis of *Trichophyton species* by using multiplex PCR , the result showed only 27 isolates gave positive for specific genes which included 16 (59.26%) of *Trichophyton mentagrophytes* , 7 (25.93%) of *Trichophytin rubrum* and 4 (14.81%) of *Trichophyton violaceum*.

On the other hand, Virulent factors are also investigated the molecular detection of *phospholipase gene* , *transcription factor PacC gene* and *heat shock protein hsp90* gene by polymerase chain reaction technique showed that all 27 (100%) *Trichopyton* isolates gave positive results for these marker, the *keratinase gene* was also detedcted and the result was shown that gene was present in *Trichophyton mentagrophytes* only 15 isolates gave potitive but in *Trichophytin rubrum and Trichophyton violaceum* were showed that all 11 isolates gave positive .

In this study, multilocus sequence typing method (MLST) were used to investigate the discriminatory ability, reproducibility, and the genetic relationship between 15 *Trichophyton species* isolates. The sequence data obtained for MLST for determining the population structures analyzing the extent of linkage disequilibrium between alleles for phylogenetic relationships between 15 isolates, depended on the seven housekeeping genes frequently used for MLST analysis of *Trichophyton species* (*ITS , BT2 , TEF-1α, ACT , HSP70 , D1/D2 and CaM*) .

The mean guanine cytocin content of sequnces of seven gene fragments ranged from 48%(*CaM* and *HSP70*) to 57 %(*ITS* and *D1/D2*):Trimmed fragment size of the 7 selected loci ranged from 399 b-p (HSP70) - 767 b-p (BT2).

The nucleotide diversity ranging from 0.0004 to 0.05600 pergene . Moreover , The number of polymorphic sites per locus varied between 2 (CaM) -128(TEF-1 α).

According to allelic profile it was found that the presence of variant (SNP, insertion, or deletion) between isolates. In the case HSP70 was more variant or mutant than other 6 housekeeping genes, contrary to the CaM which was the least variant. The gene chosen for the present MLST scheme seem to be representatives of the general polymorphism seen in house keeping genes of Trichophyton species

The DNA sequence were aligned and analyzed for each gene fragments. The phylogeny of these ST, an MLST phylogenetic tree of all the *Trichophyton species* strains was inferred maximum like hood approach from concatenated sequence. All *Trichophyton species* isolates showed polyphyletic lineage and revealed two distinct clusters, cluster A contain 13 isolates and cluster B contain only two isolates .

The split graphs for the seven gene (*ITS , BT2 , TEF-1α, ACT , HSP70 , D1/D2 and CaM*) revealed network like with parallelogram structures indicating

that intergenic recombination had occurred during the evolutionaty history of these genes . However ,the split graphs of HSP70 are tree like structures suggesting that the descent of these genes was clonal and absence of recombination . The split decomposition analysis of combined seven MLST Loci display network like structure with rays of different length.

At a genomic level, this study tried to discriminate between closely related strains, *Trichophytone mentagrophytes* (6TR ,7TR) , by using whole genome sequencing . The results of this study noted the chromosomes of *trichophytone mentagrophytes* (6TR ,7TR) are very differences , Regarding the first raw data in *Trichophyton mentagrophytes* (6TR) , A 683.778614 M of total nucleotides bases were produced and total sequences were 4.531292 M. The GC content was 49.06% .*Trichophyton mentagrophytes* (7 TR) , A 2.443309 G of total nucleotides bases were produced and total sequences were 16.319284 M . After comparative genome analysis , all the studied genomes of *Trichophyton mentagrophytes* (6TR and 7RT) showed different patterns of evolutionary events (genomic rearrangements and segmented gain or loss) with each other or with the reference genome .

In addition , the sequence similarity of *Trichophyton mentagrophytes* was studied in comparison with the reference genome , the result of this study revealed that 7 TR strain resulted more CDS than 6TR. Moreover, genome of 7TR strain possessed more genes encoded for ORF and rRNA than other studied strain. Moreover, the higher number of pseudogenes were identified in 7TR strain rather than 6TR strain indicating to gain new genes with non-functions. Also , the results of this study revealed that most of these proteins were identified gene ontology protein more than other proteins that were mapped to KEGG pathways , protein with uniport or protein with compartments .

Genome annotation of gene ontology (GO) and Kyoto Encyclopedia of gene and geneme (KEGG)was also studies and the result was show tha GO were categories into three main GO categorise which include molecular function ,cellular component and biological process . According to KEGG analysis , 9370 in (6TR) and 9099 in (7TR) were annoted and assigned to different KEGG which could be classified into six main KEGG categories : genetic information process , environmental informal processing , cellular processes , metabolism ,human diseases and organismal system

Carbohydrate active enzyme (CAZy) families are divided in to five classes , glycoside hydrolysis (GHs), carbohydrate estrases (CEs) ,glycotransferases (GTs) , Auxiliary activities (AA) and carbohydrate binding module (CBM) .Annotation of the predicated amino acid sequence using (CAZy) data base of *Trichophyton mentagrophytes* (6TR ,7TR) revetated 46% GHs ,42 % GT ,6 % AA ,5% CBM and 1% CEs.

The anti SMASH show that the 6TR ,7TR isolates have atotal 22 and 24 biosynthesis gene cluster (BGC) respectively , suggesting that it has agreat potential to produce a wide variety of secondary metabolisim .

Furthermore, the results of this revealed that SNPs variants covered the higher number of variant counts in all studied genomes (6TR and 7TR), where 70362 (97.65%) of 6TR variants identified as SNPs while only 687 (0.95%) and 1006 (1.4%) of the total number of 6TR variants detected as Insertions and Deletions. Similarly, of a total of 629423 variants determined in 7TR genome, SNPs comprised 565184 (89.79%) while only 32667 (5.19%) Insertions and 31572 (5.02%) Deletions were identified .

The most common patterns of base substitution in all studied genome were C ↔ T and G ↔ A substitutions in 7TR. While in 6TR the most common patterns

of base substitution in studied genome were A↔ C and T ↔ G Based on these results, these patterns represent Transition substitution rather than Transversion substitution, where Transition variants comprised the higher percentages in comparison with Transversion variants in all studied genomes after SNPs analysis. In conclusion , the results of this study provide a comprehensive frame work for under standing the whole genome of *Trichophyton mentagrophytes* .

The phylogenetic tree showed that the isolates from this study were tightly clustered together as one group . The *Trichophyton mentagrophytes* isolates from different infection close genetic relationships , and epidemiology or trace back evidence was provided to establish the connection between these *Trichophyton mentagrophytes* isolates.

phylogenetic analysis of the studied *Trichophyton mentagrophytes* genomes (6TR and 7TR) was performed to determine the closest representative genomes of *Trichophyton mentagrophytes* TIMM 2789. This strain shared high homology with studied 6TR and 7TR strains , and grouped with the same clade for each one suggesting same clone origin

الخلاصة

خلال فترة الدراسة تم جمع 150 عينة (مريض) تشمل جميع الأعمار ومن كلا الجنسين الذين تم تشخيص إصابتهم بالتهاب فطري جلدي في عيادة الأمراض الجلدية، تم اخذ العينة من خلال كشط الجلد (عدد = 100) والأظافر (عدد = 50)، تمت زيارة المرضى لعيادات الخارجية للأمراض الجلدية في مستشفى مرجان التعليمي والعيادة الخارجية للأمراض الجلدية في مستشفى الصادق التعليمي، وتم تشخيص هذه العينات من قبل أطباء الامراض الجلدية في الفترة ما بين نيسان وتشرين الاول عام 2022. وتم تشخيصها باستخدام اختبارات الزراعة والكيمياء الحيوية واستخدام Primes محدد

العينات (150) التي تم جمعها خلال الدراسة تم عزل 50 عينة فقط من أنواع *Trichophyton* المعزولة عن طريق الفحص المجهري المباشر (10% هيدروكسيد البوتاسبوم) أو الزراعة على وسط الزرعيه واختبار الكيمياء الحيوية. وتم التأكد من تشخيص أنواع *Trichophyton* باستخدام PCR حيث أظهرت النتائج أن 27 عزلة فقط أعطت إيجابية لجينات نوعية شملت 16 (59.26%) من *Trichophyton mentagrophytes* و 7 (25.93%) من *trichohpyton rubrum* و 4 (14.81%) من trichophyton violaceum

استخدم الكشف الجزيئي لدراسة العوامل الضراوة من خلال الكشف عن جين الفسفوليباز وجين عامل النسخ (PacC) وجين بروتين الصدمة الحرارية (hsp90) بتقنية تفاعل البوليميراز المتسلسل وأظهرت أن جميع عزلات *Trichophyton* والتي عددها 27 أعطت نتائج إيجابية ، بينما أظهرت النتيجة وجود جين الكيراتيناز في *Trichophyton mentagrophytes* المعزولة فقط 15 عزلة وجوده في جميع العزلات *trichohpyton rubrum* و *trichophyton* violaceum الـ 11 عينة

استخدمت طريقة الكتابة التسلسلية متعددة البؤر (MLST) للتحقق من القدرة التمييزية، والتكاثر، والعلاقة الوراثية بين 15 عزلة. تعتمد بيانات التسلسل التي تم الحصول عليها لـ MLST لتحديد الهياكل السكانية التي تحلل مدى اختلال الارتباط بين الأليلات للعلاقات التطورية بين 15 عزلة، على جينات Housekeeping sequence السبعة المستخدمة بشكل متكرر لتحليل MLST *ITS* و *BT2* و *TEF-1α* و *ACT* و *HSP70*, و *D1/D2* و *CaM*

اظهرت الدراسة ان متوسط محتوى CG لتسلسلات سبع جينات من 48% (CaM وHSP70) إلى 57% (ITS وD1/D2): تراوح حجم الشظية المشذبة للمواقع سبع جينات من 399 وزج قاعدي HSP70 الى 767 زوج قاعدي BT2 .

وتنوع النيوكليوتيدات من 0.0004 إلى 0.05600 لكل جين . بالإضافة الى تراوح عدد المواقع متعددة الاشكال لكل موقع بين 2 (*TEF-1 α*) - 128(*CaM*).

حددت خمسة عشر نوعًا من التسلسلات(STs)، اذ أظهر ان جميع العزلات هي سلالة متعددة الأصول ، مما يعني أنها لم تنحدر من سلف مشترك واحد. تم تقسيم العزلات إلى مجموعتين متميزتين: مجموعة(أ) ومجموعة (ب). المجموعة (أ) تحتوي على عزلة واحدة تم تقسيم 13 عزلة من هذه المجموعة إلى عناقيد فرعية ، بينما قسمت المجموعة (ب) إلى فرعين وكل فرع يحتوي على 2 عزلتين

وفقا للملف الاليلى وجد متغير اليلي (SNP، الحشر أو الحذف) بين العزلات في حالة HSP70 كان أكثر تنوعًا أو طفرة من جينات Housekeeping sequence الستة الأخرى، على عكس CaM الذي كان الأقل تنوعًا. يبدو أن الجين المختار لمخطط MLST الحالي يمثل تعدد الأشكال العام الذي يظهر في جينات الحضانة المنزلية لأنواع *Trichophyton*

تسلسل الحمض النووي تم تحليل كل أجزاء الجينات فيه . أظهرت شجرة التطور والنشوء ان جميع عزلات الفطريات *Trichophyton* نسباً متعدد العرقيات وكشفت عن مجموعتين متميزتين، المجموعة A تحتوي على 13 عزلة والمجموعة B تحتوي على عزلتين فقط.

كشفت الرسوم البيانية للجينات السبعة (*ITS* و*BT2* و*TEF-1α* و*ACT* و*HSP70* و*D1/D2* و*CaM*) عن شبكة تشبه هياكل متوازي الأضلاع مما يشير إلى أن إعادة التركيب بين الجينات قد حدثت خلال تاريخ تطور هذه الجينات. ومع ذلك، فإن الرسوم البيانية لـ HSP70 هي هياكل شبيهة بالشجرة مما يشير إلى أن نزول هذه الجينات كان نسيليًا وغياب إعادة التركيب . التحليل لسبع شبكات عرض MLST Loci مجتمعة مثل الهيكل بأشعة ذات اطوال مختلفة.

المستوى الجينومي ، حاولت هذه الدراسة التمييز بين السلالات ذات الصلة *Trichophytone* (6TR ,7TR) *Mentagrophytes* باستخدام التسلسل الجينومي الكامل. أشارت نتائج هذه الدراسة إلى أن كروموسومات *Trichophytone Mentagrophytes* (TR ,7TR6) تختلف بشكل كبير ، وفيما يتعلق بالبيانات الأولية في *Trichophytone Mentagrophytes* (

TR6) فقد تم إنتاج 683.778614 ميكا من قواعد النيوكليوتيدات الكلية وكان إجمالي التسلسلات 4.531292 ميكا . بلغت نسبة المحتوى 49.06% .في *Trichophytone Mentagrophytes* (TR7) تم انتاج 2.443309 كيكا من قواعد النيوكليوتيدات الكلية وبلغت المتتابعات الكلية 16.319284 م . بعد تحليل الجينوم المقارن، أظهرت جميع الجينومات المدروسة *Trichophytone Mentagrophytes* (TR6 وRT7) أنماطًا مختلفة من الأحداث التطورية (إعادة ترتيب الجينوم والكسب أو الخسارة المجزأة) مع بعضها البعض أو مع الجينوم المرجعي .

تمت دراسة التشابه التسلسلي *Trichophytone Mentagrophytes* بالمقارنة مع الجينوم المرجعي، وأظهرت نتائج هذه الدراسة أن سلالة TR 7 تحتوي على CDS أكثر من TR6. علاوة على ذلك، يمتلك جينوم سلالة TR7 عددًا أكبر من الجينات المشفرة لـ ORF وrRNA مقارنة بالسلالات الأخرى المدروسة. علاوة على ذلك، تم تحديد العدد الأكبر من الجينات الكاذبة في سلالة TR7 بدلاً من سلالة TR6 مما يشير إلى اكتساب جينات جديدة ذات وظائف غير وظيفية. كما كشفت نتائج هذه الدراسة أن معظم هذه البروتينات تم التعرف عليها كبروتينات جينية أكثر من البروتينات الأخرى التي تم تعيينها إلى مسارات KEGG، البروتين مع uniport أو البروتين

تمت أيضًا دراسة شرح الجينومي لعلم الجينات (GO) وموسوعة كيوتو للجينات (KEGG) وكانت النتيجة إظهار أن GO كانت مقسمة إلى ثلاث فئات GO رئيسية والتي تشمل الوظيفة الجزيئية والمكونات الخلوية والعملية البيولوجية. وفقا لتحليل KEGG، تم شرح 9370 في (TR6) و9099 في (TR7) وتعيينها إلى KEGG مختلفة والتي يمكن تصنيفها إلى ست فئات رئيسية: عملية المعلومات الجينية، والمعالجة البيئية غير الرسمية، والعمليات الخلوية، والتمثيل الغذائي، والأمراض البشرية والنظام العضوي .

تنقسم عائلات الإنزيمات النشطة للكربوهيدرات (CAZy) إلى خمس فئات، التحلل المائي للجليكوسيد (GHs)، واسترات الكربوهيدرات (CEs)، والناقلات السكرية (GTS)، والأنشطة المساعدة (AA) ووحدة ربط الكربوهيدرات (CBM). شرح للحمض الأميني الأساسي تم إعادة تنشيط التسلسل باستخدام قاعدة بيانات (CAZy) *Trichophyton* (TR6،7TR) بنسبة 46% GHs، 42% GT، 6% AA، 5% CBM و1% CEs.

يُظهر Anti SMASH أن العزلات TR6 وTR7 تحتوي على إجمالي 22 و24 مجموعة جينية للتخليق الحيوي (BGC) على التوالي، مما يشير إلى أن لديها إمكانات كبيرة لإنتاج مجموعة واسعة من الأيض الثانوي .

علاوة على ذلك، كشفت نتائج هذا أن متغيرات SNPs غطت العدد الأكبر من أعداد المتغيرات في جميع الجينومات المدروسة (TR6 وTR7)، حيث تم تحديد 70362 (97.65٪) من متغيرات TR6 على أنها SNPs بينما تم تحديد 687 فقط (0.95٪) و1006 (1.4٪). %) من العدد الإجمالي لمتغيرات TR6 التي تم اكتشافها كعمليات إدراج وحذف. وبالمثل، من إجمالي 629423 متغيرًا تم تحديدها في جينوم TR7، تتألف أشكال SNP من 565184 (89.79٪) بينما تم تحديد 32667 (5.19٪) فقط من عمليات الإدراج و31572 (5.02٪) من عمليات الحذف.

كانت الأنماط الأكثر شيوعًا لاستبدال القاعدة في جميع الجينوم المدروس هي بدائل T ↔ C و A ↔ G في TR7. بينما في TR6 كانت الأنماط الأكثر شيوعًا لاستبدال القاعدة في الجينوم هي A↔ C وG ↔ T. وبناءً على هذه النتائج، تمثل هذه الأنماط استبدال الانتقال بدلاً من استبدال التحويل، حيث شكلت متغيرات الانتقال النسب المئوية الأعلى مقارنة بمتغيرات التحويل في جميع دراسة الجينومات بعد تحليل SNPs. في الختام فإن نتائج هذه الدراسة توفر إطار عمل شامل لفهم الجينوم الكامل *Trichophytone Mentagrophytes*.

أظهرت شجرة النشوء والتطور أن العزلات من هذه الدراسة كانت متجمعة بشكل محكم كمجموعة واحدة. تعزل عزلات *Trichophyton mentagrophytes* من عدوى مختلفة العلاقات وراثية ، وقد تم تقديم الأدلة الوبائية لإثبات العلاقة بين عزلات *Trichophyton mentagrophytes* .

كذلك تم إجراء تحليل النشوء والتطور لجينومات *Trichophyton mentagrophytes* (TR6 وTR7) لتحديد أقرب الجينومات *Trichophyton mentagrophytes* TIMM 2789. تشترك هذه السلالة في تماثل عالٍ مع سلالات TR6 وTR7 ، وتم تجميعها مع نفس الفرع الحيوي لكل واحدة مما يشير إلى نفس الاصل

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List of Abbreviation

Abbreviation	*Meaning*
WHO	World Health Organization
NHD	Neglected Tropical Diseases
MLST	Multiple locus sequence typing
ST	Sequence type
PCR	Polymerase chain reaction
WGS	Whole-genome sequencing
SDA	Sabouraud dextrose agar
PDA	Potato Dextrose Agar
MIC	Minimum inhibitory concentration
KOH	Motassium hydroxide
ISHAM	International Society for Human and Animal Mycology
ITS	Internal Transcribed Spacer
MALDITOFMS	Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry
RAPN	Random amplification of polymorphic DNA
HSP	Heat shock protein
BT 2	Beta-tubulin
TEF1-a	Rranslation elongation factor 1-a
CaM	Calmodulin
SNP	Single nucleotide polymorphism
MLEE	Multilocus enzyme electrophoresis
NGS	Next generation sequencing

MLST	Multiloucs sequence type
WTS	Whole transcriptome sequencing
MES	Methylation sequencing
WES	Whole-exome sequencing
CGS	Candidate gene sequencing
SOLID	Sequencing by Oligonucleotide Ligation and Detection
SMRT	Single-molecule realtime sequencing
LCB	lactophenol cotton blue
DTM	dermatophyte Test Medium
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
MtDNA	Mitochondrial DNA
RAPD	Random amplification of polymorphic DNA
HSP	Heat shock protein
ST	Sequence type
SNP	Single nucleotide polymorphism
TBE	Tris-Borate-EDTA
BCP	Bromocresol purple
GO	Gene ontology
KEGG	Eukaryotic Orthologous Groups
CAZy	Carbohydrate-Active Enzyme
LCBs	locally collinear blocks
tRNA	Transfer RNA
rRNA	Ribosomal RNA

SSR	Simple sequence repeat
ORF	Open Reading Frame
CDS	Coding sequences
GHs	Glycoside hydrolysis
CEs	Carbohydrate estrases
GTs	Glycotransferases
AA	Auxiliary activities
CBM	Carbohydrate binding module
PL	Polysaccharide lyases
LPMOs	lytic polysaccharide monooxygenases
BGC	biosynthetic gene cluster
NPRS	Non-Ribosomal Peptide Synthetase
T1PKS	Type I Polyketide Synthase
SMs	Secondary metabolites
SAPs	single amino-acid polymorphisms
PFGE	pulsed-field gel electrophoresis
anti SMASH	Antibiotics and Secondary Metabolite Analysis Shell

Chapter One

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1.1 Introduction

Trichophyton is a genus of fungi within the group of dermatophytes, which are specialized fungi that cause infections of the skin, nails and hair in humans . *Trichophyton species* are among the most common causative agents of dermatophytosis, also known as ringworm infections (Li *et al* ., 2021).

There are numerous species within the *Trichophyton* genus, and they are classified based on their morphological and physiological characteristics, as well as molecular analysis. Some well-known species include *Trichophyton rubrum, Trichophyton mentagrophytes and Trichophyton violaceum*.

Dermatophytosis caused by *Trichophyton species* can manifest in various clinical forms depending on the site of infection. These can include ring-shaped skin lesions, scaling, itching, redness, and thickening of the affected area. *Trichophyton* infections are typically transmitted through direct contact with infected individuals or contaminated surfaces, such as floors, towels, or shared personal items (Ianiri *et al*., 2018).

Trichophyton infections can manifest in different forms depending on the site of infection including tinea corporis ,tinea pedis ,tinea cruris ,tinea capitis and tinea unguium .

Trichophyton species are characterized by their ability to invade and utilize keratinized tissues such as the skin, nails and hair as a nutrient source. They produce specialized enzymes called keratinases, which can break down and degrade keratin, the main protein component of these tissues. This ability allows them to colonize and cause infections in the host (Mariyammal *et al* ., 2018).

In culture, *Trichophyton species* exhibit several general characteristics ,*Trichophyton* colonies on culture media such as Sabouraud dextrose agar (SDA) typically appear cottony, fluffy, or powdery , growth rate exhibit moderate to rapid growth on appropriate culture media. And The texture of *Trichophyton* colonies

can be velvety or granular, often display a different color on the reverse side of the agar compared to the top surface (Alex *et al* ., 2022).

On the other hand , *Trichophyton species* can be distinguished from other dermatophytes through various biochemical and physiological characteristics like urease activity ,hair perforation test and nitrate utilization . It's important to note that specific characteristics and biochemical tests may vary among different *Trichophyton species* (Chander , 2009) .

However , *Trichophyton species* produce a range of virulence factors that enable them to invade and colonize host tissues. These include phospholipase , transcription factor , heat shock protein 90, and keratinases, which help the fungi to digest and penetrate host tissues (Mingrui *et al*., 2019) .

Keratinases are proteolytic enzymes that specifically target and hydrolyze keratin substrates. They play a crucial role in the pathogenesis and virulence of *Trichophyton species* by facilitating the invasion and colonization of keratinized tissues (de Menezes *et al* ., 2021).

Phospholipases are enzymes that hydrolyze phospholipids, which are major components of cell membranes. By breaking down phospholipids, phospholipase enzymes can disrupt the integrity of host cell membranes, facilitating the invasion of fungal pathogens into host tissues (Zuzarte *et al* ., 2021).

The *PacC gene* encodes the PacC transcription factor, which acts as a master regulator of pH-responsive gene expression. The protein is typically synthesized as an inactive precursor that is proteolytically processed and activated in response to alkaline pH conditions. Once activated, PacC translocates to the nucleus and binds to specific DNA sequences, thereby modulating the expression of target genes involved in pH adaptation, stress response, virulence, and other cellular processes (Ferreira *et al* ., 2006).

The HSP90 protein, which functions as a molecular chaperone. HSP90 assists in the folding, stabilization, and activation of a wide range of client proteins, many of which are key regulators of cellular processes. It plays a critical role in maintaining protein conformational integrity under normal conditions and is also upregulated in response to various stressors, including heat shock, oxidative stress, and exposure to antifungal drugs (Kumawat *et al* ., 2022).

Multilocus sequence type has proven to be a valuable tool in the molecular epidemiology and population genetics studies of *Trichophyton species*. It provides a standardized and reproducible method for characterizing and comparing isolates, contributing to a better understanding of the epidemiology, evolution, and pathogenicity of these fungal pathogens (Bougnoux *et al* ., 2002).

MLST data can also help in understanding the potential virulence factors and adaptive evolution of *Trichophyton species.* By comparing the MLST profiles of clinical isolates, can identify clusters of related strains and assess their association with specific clinical manifestations or treatment outcomes (Eduardo and Miguel 2017).

Genome sequencing and comparative genomics have revealed insights into the genetic diversity and evolution of *Trichophyton species*.

Whole genome sequence provides valuable insights into their genetic makeup, evolution and pathogenicity. Sequencing whole genome of *Trichophyton* isolates, can identify and compare genetic variations within and between species. This information helps in understanding the population structure, phylogenetic relationships, and transmission patterns of *Trichophyton* strains. WGS data can reveal the genetic diversity and relatedness of isolates, enabling the identification of specific lineages or clusters associated with particular geographic regions or clinical presentations. Furthermore, WGS allows for the identification of genes

and genetic variations that are potentially involved in *Trichophyton* pathogenicity (Köser *et al* ,. 2012 **)** .

Understanding the diversity, epidemiology, and virulence factors of *Trichophyton species* is crucial for effective diagnosis and prevention of dermatophytosis.

Aim of study: -

This study was aimed to genetic diversity of *Trichophyton species* isolated from clinical specimens.

Objectives

1- Isolation and detection of *Trichophyton species* from clinical specimens (skin and nails scrapings) .
2- Molecular diagnosis of *Trichophyton species* by using specific primer.
3- Detection of some virulence factors of *Trichophyton species* .
4- Genotyping diversity of *Trichophyton species* by using Multilocus sequence type.
5- Whole Genome Sequencing of *Trichophyton* species .

1-2 Literature Review

1.2.1 General Characteristics of *Trichophyton*

Trichophyton is a genus of fungi, that includes several species of dermatophytes, which are fungi that infect the skin , nails and hair of humans which includes the parasitic varieties that cause tinea : tinea corporis (ringworm), tinea cruris (jock itch), tinea pedis (athlete's foot), and tinea capitis (scalp ringworm).The hyphae produce asexual spore known as conidia, these conidia are typically produced at the tips of specialized hyphae called conidiophores. There are two main types of conidia are macroconidia and microconidia. Macroconidia (macroaleuriospores) are mostly borne laterally directly on the hyphae or on short pedicels, and are thin- or thick-walled, clavate to fusiform, and range from 4 to 8 by 8 to 50 μm in size, blunt at the end, and with numerous transverse septa macroconidia are few or absent in many species. Microconidia (microaleuriospores) are abundant in presence small, thin walled, hyaline, sub-spherical to club-shaped and borne singly or in grape-like cluster are spherical, pyriform to clavate or of irregular shape, and range from 2 to 3 by 2 to 4 μm in size (Li *et al* ., 2021) .

These are typically transmitted through direct contact with infected individuals or contaminated objects, such as towels, clothing, or bedding . The genus *Trichophyton* is quite diverse, with over 20 recognized species. Some of the most common species that cause human infections include *Trichophyton rubrum*, *Trichophyton mentagrophytes* (Nenoff *et al* ., 2018).

Trichophyton infections can cause a range of clinical manifestations, depending on the site of infection and the species involved. Common symptoms include itching, redness, scaling, and hair loss. In severe cases, secondary bacterial infections may also occur.

Trichophyton species can be grown on various types of culture media, including Sabouraud dextrose agar (SDA), which is a common medium used for the isolation and identification of dermatophytes . It contains a low pH (around 5.6) and a nutrient-rich mixture of peptone, dextrose, and agar, which provides a supportive environment for fungal growth (Adjapong *et al*., 2014).

On SDA, *Trichophyton species* typically form flat, velvety colonies that are white to yellowish in color. The colonies may have a powdery or granular texture and may become more pigmented with age and grow relatively slowly .On SDA, taking 4 weeks to form visible colonies with chloramphenicol and cycloheximid were incubated at (25 °C ± 5 °C) , If there was no growth even after 4 weeks of incubation it was taken as negative (Alex *et al* ., 2022) .

The growth rate may be influenced by the temperature and humidity of the incubation environment. The size of *Trichophyton* colonies on SDA may vary depending on the species and the age of the colony. In general, colonies are small to medium in size, ranging from 2-10 mm in diameter also it and may be white to yellowish in color, with some species producing pigments that range from pink to reddish-brown. Also, the colonies have a characteristic texture that is often described as "cottony" or "woolly." The texture may become more granular or powdery with age .

Also , dermatophyte Test Medium (DTM) is a commonly used synthetic medium that is selective for dermatophytes like *Trichophyton*. DTM contains cycloheximide and antibiotics to inhibit the growth of bacteria and saprophytic fungi (De Hoog *et al* ., 2008) .

Corn meal agar is a type of nutrient-rich medium that is commonly used for the cultivation of various types of fungi, including dermatophytes like *Trichophyton*. Corn meal agar contains a variety of nutrients that can support the growth of many different types of fungi. These include amino acids, vitamins, and

minerals that are essential for fungal growth and metabolism (De Hoog *et al* ., 2008) .

On the other hand , Growth on Potato Dextrose Agar (PDA) This medium is used in microbiology for the cultivation of fungi. Its nutrient-rich composition makes it ideal for supporting the growth of a wide range of fungal species. When inoculated with fungal spores or mycelial fragments, PDA provides the necessary nutrients and conditions for fungal growth and sporulation (Hiba and Mouna , 2022).

The branching hyphae are produced by *Trichophyton* in the artificial culture and nonparasitic environmental state .Sometimes abnormal forms of hyphae such as spiral or coiled hyphae, tennis racquet hyphae, pectinate hyphae (like teeth of a comb) and 'favic chandeliers' (irregular projections along one side of the hyphae) are observed . In the parasitic state, *Trichophyton* produces hyphae and arthroconidia (Indranil *et al*., 2015).

Differentiate *Trichophyton* from other fungi, additional diagnostic tests may be performed, such as microscopic examination of fungal structures by used direct microscopy of skin scrapings or nail using potassium hydroxide (KOH) or fungal stains, such as phenol cotton blue and biochemical tests to identify specific enzymes produced by the *Trichophyon* including urease test , nitrate reduction test , tween 80 hydrolysis test, acid production test , indole production test and hair perforation test (Chander , 2009).

It should be noted that while biochemical tests can be useful for differentiating *Trichophyton species*, they are not always definitive and should be used in conjunction with other diagnostic methods, such as molecular techniques (Nenoff *et la* ,. 2018) .

Polymerase chain reaction (PCR) is a molecular technique that can be used to detect *Trichophyton species* in clinical samples. PCR amplifies specific regions of

fungal DNA, allowing for rapid and sensitive detection of the fungus (Badali *et al* ., 2018) .

PCR-based methods have become an important tool for the diagnosis and management of dermatophytosis, particularly in cases where conventional methods, such as microscopy and culture, have limitations. PCR methods offer sensitive and specific tools for identify *Trichophyton species* , which can help in understanding the ecology and epidemiology of the fungus (Badali *et al* ., 2018) .

Trichophyton is rarely life-threatening, it is chronic, relapsing, and they can easily transmit from one patient to the another and from environment to human significantly affect the patients quality of life (Fekrazad *et al*.,2017) .

This pathogen particularly affects the immunocompromised patients, including those who are undergone long-term steroid therapy, bone marrow or solid organ transplantation, patients with diabetes mellitus and also known multifactorial risk factors, including overcrowding, poor hygiene, cultural habits, heat, humidity, socioeconomic status and migration of people and involvement of workers in different occupational activities like farming, athletes . Prevalence rates and culprit organisms widely differ among regions of the world and are related to gender, age, climate, demographic, and socio-economic factors (Segal *et al* ., 2021) .

And other risk factors for extensive dermatophytosis include genetic defects , misdiagnosis or delayed diagnosis (Mingrui *et al* ., 2019).

Dermatophytosis is a significant public health problem worldwide, particularly in tropical and subtropical regions, where it is highly prevalent. The World Health Organization estimates that dermatophytosis affects over 20% of the world's population, with higher rates reported in low- and middle-income countries. In addition, dermatophytosis can lead to secondary bacterial infections, which can further complicate treatment and increase morbidity. Moreover, dermatophytosis is highly contagious and can spread rapidly in crowded settings,

such as schools and prisons .Prevention of dermatophytosis involves avoiding contact with infected individuals or animals, as well as practicing good hygiene. This includes washing hands and feet regularly, keeping skin and nails clean and dry, and avoiding sharing personal items, such as towels, clothing, and shoes. In addition, treating pets with antifungal medications can help prevent the spread of infection from animals to humans (Gupta *et al* ., 2021).

1.2.2 Taxonomy and Nomenclature of *Trichophyton*

Trichophyton is a genus of dermatophytic fungi that includes several species that can cause various forms of dermatophytosis in humans . The taxonomy and nomenclature of *Trichophyton* have undergone several revisions over the years based on advances in molecular techniques and phylogenetic analysis (Gräser and de Hoog , 2018) .

The history of the classification of *Trichophyton*, including the original description of the genus by Robin in 1853. The taxonomy of *Trichophyton*, which includes several species that are differentiated based on morphological, ecological, and genetic characteristics.

The latest taxonomic framework for *Trichophyton*, as proposed by the International Society for Human and Animal Mycology (ISHAM) in 2017, recognizes 20 species within the genus according to phylogenetic analysis of DNA sequences from multiple genes (Dukik *et al* ., 2017).

The taxonomy of *Trichophyton* is important for the appropriate diagnosis and treatment of dermatophytosis, as different species may have different susceptibilities to antifungal drugs and may respond differently to treatment. Accurate identification of *Trichophyton species* can also aid in understanding the epidemiology and ecology of the fungus(Dukik *et al* ., 2017) .

The taxonomy of Trichophyton

Kingdom: Fungi

Phylum: Ascomycota

Class: Euascomy

Order: Onygenales

Family: Arthrodermataceae

Genus: *Trichophyton* 20 species

The genus *Trichophyton* is placed in a derived position on the evolutionary tree and contains zoophilic and anthropophilic and geophilic species. The rRNA intergenic transcribed spacer region (i.e. ITS1, 5.8S rRNA and ITS2) is the most informative marker available for this genus (Gräser *et al*., 2000) .

Species within the genus *Trichophyton* are closely related, with only small genetic distances between them. Interestingly, the low genetic variation of the ITS region in the *T. rubrum* complex contrasts with a high phenotypic variability. This description of taxa that were later synonymized with the current three species: *T. rubrum (including T. raubitschekii, T. kanei, T. fischeri, T. flavum, T. fluviomuniense, T. pedis, T. rodhainii, T. kuryangei and T. megninii), T. soudanense (including T. circonvolutum and T. gourvilii)* and *T. violaceum* (including *T. yaoundei, T. glabrum and T. violaceum var. indicum)* , the genetic distance between the ITS region of *T. rubrum and T. violaceum* is sufficiently large to recognize them as separate species. The distance between *T. rubrum* and *T. soudanense* is smaller, but a polyphasic approach, in which a phylogenetic analysis of the ITS gene was combined with data on morphology, physiology, geography and clinical characteristics, has led to the recognition of *T. soudanense* as a separate (Gräser *et al* ., 2008) .

On the other hand , *T. mentagrophytes* has been identified and recognised with the help of sequencing of the ITS region of the rDNA. However, led to a basic confusion owing to the newly introduced taxonomy of dermatophytes in 2017. According to this most recently suggested classification and new taxonomy of dermatophytes, the former "*T. mentagrophytes* complex" is differentiated into *T. mentagrophytes* (zoophilic strains) and *T. interdigitale* (anthropophilic strains).

On the other hand , the *Trichophyton mentagrophytes* species complex are common agents of human dermatophytosis which invading skin layers and causing superficial infections, such as tinea corporis or athlete's foot , with an increased incidence since 1965. The species included in this anthropo-zoophilic complex, which were first defined on the basis of morphological features and mating-type studies, have more been investigated using molecular methods revealing an organization more complex than expected (Kardjeva *et al* ., 2006).

Several other methods, such as PCR fingerprinting and Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) were indeed unable to distinguish all accepted species within the complex, demonstrating again their close interrelationships species (Packeu *et al* ., 2020).

The mentioned molecular approaches, such as G+C content analysis, total DNA homology, restriction fragment length polymorphism (RFLP), analysis of mitochondrial DNA (mtDNA), random amplification of polymorphic DNA (RAPD) analysis, and PCR fingerprinting, were likely employed to investigate the genetic diversity and relationships among different *Trichophyton species* (Faggi *et al* ., 2001).

1.2.3 *Trichophyton species*

1.2.3.1 *Trichophyton mentagrophytes*

Trichophyton mentagrophytes is species of dermatophyte that can cause skin, and nail infections in humans . It is a zoophilic fungus, meaning that it primarily infects animals .It is associated with infections in cats, dogs, and horses but can also cause infections in humans. It is commonly found in the soil and is transmitted from animals to humans through direct contact or by contact with contaminated objects. In humans, *T. mentagrophytes* infections can affect various parts of the body, including the scalp, skin, nails, and feet.

The clinical manifestations of *T. mentagrophytes* including athlete's foot, ringworm, and nail infections. The symptoms can vary depending on the site of infection but often include itching, scaling, redness, and inflammation (Gupta and Stec 2019).

T.mentagrophytes is a contagious fungus which primarily causes dermatophytosis such as tinea pedis, tinea unguium, tinea corporis, and tinea capitis. It distributes all over the world, especially in moist and carbon-rich environments. Colonies of *T. mentagrophytes* are generally characterized by flat, white to cream in color, with a powdery to the granular surface. Reverse pigmentation is usually a yellow-brown to reddish-brown color. Some cultures show central folding or develop raised central (Rezaei *et al* .,2016).

The granular colony form typically has a powdery appearance due to numerous single-celled microconidia (spores) formed. Microconidia of *T. mentagrophytes* are hyaline, smooth-walled, spherical to subspherical in shape, and occasional pyriform to clavate or of irregular shape. They are clavate to fusiform, and thin- or thick-walled with 4-5 cells separated by parallel cross-walls, which are laterally

directly grown on the hyphae or on short pedicels and Hydrolysis of urea and Hair perforation test were positive.

Outbreaks of *Trichophyton mentagrophytes* can occur in settings where there is close contact between individuals, such as in schools, sports teams, and prisons. The fungus can be spread through direct contact with an infected person or animal, or indirectly through contact with contaminated surfaces like clothing, towels, or sports equipment (Havlickova *et al* .,2008).

Trichophyton mentagrophytes infections can be associated with immunity in a few different ways : First, individuals with a weakened immune system, such as those with HIV/AIDS, diabetes, or taking immunosuppressive medications, are more susceptible to infections. In these cases, the immune system may not be able to mount an effective response against the fungus, leading to a more severe or persistent infection. Second, *Trichophyton mentagrophytes* infections can trigger an immune response in otherwise healthy individuals. When the fungus infects the skin, the body's immune system recognizes it as a foreign invader and mounts an inflammatory response. This can lead to redness, itching, and other symptoms of infection (Leung *et al* ., 2018) .

1.2.3.2 *Trichophyton rubrum*

Trichophyton rubrum is the other agent of dermatomycoses isolated from humans. It is one of the common causes of dermatophytosis, which are fungal infections of the skin, and nails in humans (Zhang and Liu 2021) .

It was first described as *Epidermophyton rubrum* by Castellani in 1910. It has been considered as a cause of chronic tinea corporis in the late nineteenth century. Since then, *T. rubrum* has spread throughout the world as the etiological agent of tinea unguium and tinea corporis .It is anthropophilic saprotroph that inhabits the upper layers of dead skin, which primarily causes dermatophytosis as tinea pedis,

tinea unguium, tinea corporis, and tinea capitis worldwide . Two types of *T. rubrum* commonly described are *T. rubrum* downy type and *T. rubrum* granular type .The downy type is formed by slender, clavate or club-shaped microconidia and does not make macroconidia under the microscope (Nenoff *et al* ., 2014) .

The granular type makes a large number of clavate (oblong with thick ends) to pyriform (pear-shaped) microconidia and scanty to moderate macroconidia. Macroconidia are usually absent, but once present, they are smooth, thin-walled multiseptate, slender and cylindrical to bacillus-shaped (Gräser *et al* ., 2008).

It is characterized by septate hyphae, which are thread-like structures that make up the body of the fungus. The hyphae of *T. rubrum* grow into the skin and nails, and can cause a variety of symptoms, including itching, redness, scaling, and inflammation (Ilkit ,2014) .

Colonies of *T. rubrum* are white and cottony on the surface and have a reverse side that ranges from yellow-brown to wine-red. Most cultures have been identified to be granular strains, which include numerous microconidia and small spores produced from asexual reproduction (Zhan 2018) . Older cultures may show numerous chlamydospores with a few clavate to pyriform microconidia (de Hoog *et al*. 2017).

On the other hand , it was unable Hydrolysis of urea and negative for Hair perforation test

The clinical manifestations of *T. rubrum* infections can vary depending on the location and severity of the infection. For example, athlete's foot is a common manifestation of *T. rubrum* infection that affects the skin between the toes, while ringworm can cause circular lesions on the skin. Nail infections caused by *T. rubrum* can lead to thickening, discoloration, and deformity of the nails (Gupta *et al* ., 2018) .

Identification of *T. rubrum* is important for effective treatment of dermatophytosis. Traditional methods of identification rely on the morphology and growth characteristics of the fungus, but molecular techniques, such as DNA sequencing, are increasingly being used for accurate identification and classification of *T. rubrum* strains (Li *etal* .,2021).

It is a ubiquitous fungus that is found in many different environmental sources, including soil, dust, and animal fur. It can be transmitted from person to person or from animal to person, and is particularly common in warm and humid regions .Also , it is able to evade the host immune system by modifying the surface of its cell wall. This allows it to avoid detection and clearance by immune cells, and can contribute to the persistence of *T. rubrum* infections (Ilkit , 2014) .

Infection lesions on the skin are usually round, erythematous, and itchy due to the inflammatory response triggered by the fungus and its metabolites. In onychomycosis, nails become thicker and separated from the nail bed; white spots and dystrophy may also occur (Peres *et al* ., 2010) .

1-2-3-3 *Trichophyton violaceum*

Trichophyton violaceum is a species of dermatophyte fungus that can cause skin infections in humans. It infections are most commonly associated with the scalp, and the fungus can also infect the nails and skin. The fungus is typically spread through direct contact with an infected person or animal, or indirectly through contact with contaminated surfaces like clothing, towels, or sports equipment . Symptoms of *Trichophyton violaceum* infection can include red, scaly patches on the skin, itching, and sometimes blistering.

Infections of the scalp can cause hair loss and scarring. The fungus is more commonly seen in tropical and subtropical regions (Queiroz *et al* ., 2016) .

It is produces septate hyphae with smooth-walled, spherical to oval-shaped conidia. The conidia are typically borne on short, lateral conidiophores.

On Sabouraud agar, It is important to note that the appearance of *Trichophyton violaceum* colonies on Sabouraud agar can vary depending on the growth conditions and the age of the culture .It colonies typically appear as flat, spreading, and velvety colonies with a purple to violet coloration. The colonies can range in size from 1 to 10 mm in diameter and may have a slightly irregular or scalloped edge. Under a microscope, the fungus typically appears as septate hyphae with smooth-walled, spherical to oval-shaped conidia . Therefore, identification of the fungus should be confirmed by a trained laboratory professional using additional diagnostic tests, such as microscopy and DNA sequencing (Kaur *et al* ., 2013) .

Outbreaks of *Trichophyton violaceum* infections are rare, but they have been reported in various parts of the world, particularly in tropical and subtropical regions. Outbreaks are more likely to occur in settings where there is close contact between individuals, such as schools and sports teams

(Da Cunha , 2017) .

1.2.4 Epidemiology of *Trichophyton*

Trichophyton are mycoses caused by pathogenic fungi that generally trigger superficial infections in humans . Infections caused by *Trichophyton* are called “tineas” and are classified according to the affected site , Infections of the scalp, called tinea capitis, are more common in children, while other tineas more commonly affect post pubertal individuals.

Trichophyton affect individuals worldwide but their incidence is higher in tropical countries because of high temperatures and humidity (Taplin *et al* 2001).

Also Factors that influence the development of dermatophytoses include age, sex, season of year, socioeconomic and cultural conditions, and geographic location (Iorio *et al*., 2007).

It is estimated that about 10 to 15% of individuals are contaminated with dermatophytes at some point in their life (Pires *et al* ., 2014).

According to data from the World Health Organization (WHO), dermatophytoses affect about 25% of the world population (Sai *et al* ., 2019) and 30 to 70% of adults are asymptomatic carriers of these diseases. Study by (Nadia *et al* ,. 2020) in Babylon province, Iraq .This study was detected dermatophytes from patients with various skin. They found that *T. rubrum* is commonly implicated in dermatophytosis and other recalcitrant human fungal diseases ,*T. rubrum* and *T. mentagrophytes* are responsible for around 27–76% and 4–41% of serious human fungal infections, respectively. However (Sudad *et la* .,2015) in Baghdad province, Iraq . they found The main etiological agents was *Trichophyton rubrum* 50% followed by *Trichophyton mentagrophytes* 32.5% .

In Western countries, 80 to 90% of onychomycosis cases are primarily caused by dermatophytes, with 5–17% being due to yeasts. In southern European countries, dermatophytes are the causative agents of 40 to 68% of cases, with 21–55% being due to yeasts. In Asian and Middle Eastern countries, dermatophytes account for 40 to 48% of cases, with 43–46% of infections being caused by yeasts (Gupta *et al* ., 2019).

The high prevalence of nail infections in North America is largely due to immigration of dermatophytes from other regions of the world such as West Africa and Southeast Asia. The global prevalence of tinea pedis is estimated at 5.5%,accounting for 50% of all cases of nail disease(Gupta and Shear 2017).

The main causative agent of tinea corporis is *T. rubrum*, which is responsible for 80 to 90% of infections. This prevalence is even higher in men and in children younger than 15 years (Piggott *et al* ., 2012).

During the last 100 years, the dermatophyte spectrum has markedly changed all over the world with differences depending on the geographic area and other factors such as migration of population. A study conducted in Sweden reported that *Trichophyton rubrum* was the main agent of fungal infection in 83% cases (Hayette and Sacheli 2015).

The risk of onychomycosis increases with age, probably due to the presence of diabetes, poor peripheral circulation, longer exposure to pathogenic fungi and suboptimal immune function. A family history of onychomycosis is another risk factor (Gupta *et al* ., 2019) .

In Brazil, epidemiological data show a higher incidence of infections caused by *T. rubrum* in the southern and southeastern regions, followed by *T. mentagrophytes*. On the other hand, a higher prevalence of *T. tonsurans, T. rubrum* are observed in the northeastern region (silva *et al* ., 2017) .

Epidemiological data show other countries on the African continent, where particularly Ethiopia, has a high number of cases of dermatophytosis, not only due to the climatic factor, but also because it is a country with low socioeconomic status, low level of hygiene and health care, and overcrowding (Araya *et al* ., 2020) .

The group of dermatophytes isolated from skin lesions changed in the seventies of the year. Befor World War II in Germany that the two types ; *M .audouinii and E .floccosum* were prevalent at the time, while the thought was the most common since the *Trichophyton rubrum* of the last century, which includes

about 90- 80% of strains and fallowing *T mentagrophytes* (Chermette *et al* ., 2008) .

Researchers have reported that high temperature and humidity level favorable for dermatophyte infection in South India elevate the growth and sporulation of *Trichophyton mentagrophytes, Trichophyton rubrum* increasing the incidence (Naglot *et al* ., 2015) .

There are several points related to the epidemiological characteristics of fungal skin diseases clinically. Tinea capitis is the most common clinical form in patients less than 12 years. Tinea cruris is more specific to men, especially young men and adolescents. Tinea pedis is not common in children (Martinez *et al* ., 2012) .

Tinea corporis and tinea capitis are the more common type of tinea which is mostly caused by *Trichophyton species* (Ali Abdul Hussein Al- Janabi *et al* .,2020).

Trichopytons are the most prevalent agents of superficial fungal infections. Excessive heat, high relative humidity, and fitted clothing have correlations to more severe and frequent disease. Specific populations can also be more predisposed to tinea corporis; for example, children. Children are also more likely to contract zoophilic infections (Nilce *et al* ., 2016) .

1.2.5 Pathogenesis of *Trichophyton*

The pathogenesis of *Trichophyton Species* is dependent on both fungal and host factors that allow the establishment of the fungus in host tissue. This occurs concomitantly with activation of the host immune system by innate defense mechanism of human skin that includes skin acidic pH, fatty acids , presence of phagocytic cells and antimicrobial peptide on skin and renewal process of skin , which aim to eliminate the pathogen. and adaptive immune cells, such as T cells

and B cells, provide long-term protection against reinfection (Martinez *et al* ., 2012) .

Several fungal virulence factors account for the establishment of infection and contribute to tissue damage, including the degradation and use of host tissue as a nutrient source.These are mainly conditions which increase of infection probability i.e humidity and temperature ,and other factors result from patients' characteristics, such as age, gender, damage of the epidermis, mechanical site of lesions, socioeconomic status, exposure to a large number of spores (Brock , 2009) .

The main steps involved in the establishment and perpetuation of *Trichophyton* infection is adhesion ,The initial step is the interaction of *Trichophyton* with the host tissues, and this adhesion to the epidermis occurs within many hour. Adhesins present on the cell wall of fungi are crucial to the initial attachment. The ability of *Trichophyon* to adhere to epithelial cells has been attributed to carbohydrate-specific adhesins, expressed on the surface of microconidia . Fibrillar projections have been observed in *T. mentagrophytes* during the adherence phase (Shyama and Thakur , 2019) .

Galactose presented on skin surface which is required for attachment (Esquenazi *et al*., 2004) . Also serine protease is also found to be a crucial player in adherence to epidermis (Baldo *et al*., 2012) .

Next ,secretion of sulfite (as a reducing agent) from sulfite efflux pump of *trichophyon* loosen the complex structure of keratin in order to facilitate the action of exo- and endo proteases . It basically cleaves cysteine-cysteine bond of keratin into cysteine and S- sulphocysteine. Then loosely structure of keratin get easily cleaved by majorly two large family of proteases that are serine proteases and metalloproteases along with leucine aminopeptidases and dipeptidyl peptidases (Burmester *et al* ., 2011) .

This is followed by germination and penetration , where arthroconidia germinate, and the hyphae quickly enter the stratum corneum to prevent elimination with cell shedding, which occurs within 3-4 hours. Between 24 hours and 3 days, the hyphae spread through the skin, The arthrocidium becomes flat, and the fibril-like structures become short and fine when it invades the deeper layers of the epidermis, leading to increased contact surface with the host tissue and thereby better adhesion and more nutrient acquisition (Monod , 2008) .

On the 4th day, the hyphae reach the granular layer, which coincides with a loss of the integrity of the epidermal barrier (Christophe , 2008) .

However, the inflammatory response activated by the presence of the fungus or release of its metabolites and virulence factors also plays a significant role in tissue damage during infection.

The fungal arsenal used to colonize host tissues comprises surface molecules responsible for attachment, secreted enzymes to convert host molecules into nutrient sources, metabolic changes to metabolize. Once enclosed in the host tissue, the pathogen must scavenge nutrients to survive while evading innate immune cells and molecules. Fungi secrete a broad spectrum of enzymes to degrade host cells, such as proteases (Nilce *et al* ., 2016) .

Several metabolic pathways are involved in fungal survival under stress conditions, such as nutrient shortages , oxidative and osmotic stresses, and exposure to antifungal drugs. These pathways allow the fungus to utilize different substrates for energy so that it may overcome the hostile environment of the host and, thus, maintain the infection process (Brock 2009) .

Trichophyton species cause cutaneous mycosis in both healthy and immunocompromised humans, with cases of deep infections associated with immunosuppression (Rodwell *et al* ., 2008) .Circulatory and metabolic disorders, like diabetes mellitus, obesity, psoriasis, hyperthidrosis, and immunosuppression,

predispose individuals to dermatophytosis, particularly tinea pedis (infection of the feet) . Genetic factors are also associated with *Trichophyton species*, mainly innate immunity deficiencies (Havlickova, 2008) .

1.2.6 Clinical manifestations of *Trichophyton species*

Trichophyton species can cause a variety of clinical manifestations, depending on the site of infection , the host immune response and the type of *Trichophyton species* (Ianiri *et al*., 2018) .

1.2.6.1 Tinea corporis

Tinea corporis is a characterized by inflammatory or non-inflammatory lesions involving areas as a reddish, scaly lesion and resolves from the center and may rapidly worsen, forming a ring-shaped, scaly or vesicle-shaped inflamed area.

The clinical manifestations result from the invasion and spread of the causative fungi in the stratum corneum of the trunk and extremities.

All known dermatophytes can produce tinea body, and the ring-like appearance characteristic of many infections ringworm is caused by the fungus moving from the center of the lesion towards the edges, forming a circular or annular shape .Tinea corporis is most often caused by *Trichophyton rubrum* and *Trichophyton mentagrophytes* (Garrett *et al* .,2023) .

Humans may become infected through close contact with an infected individual, an infected animal (in particular, domestic dog or cat), contaminated fomites and contaminated soil. Infection may be acquired as a result of spread from another site of *Trichophyton* infection (e.g.tinea capitis, tinea pedis, onychomycosis) (Leung *et al* ., 2018) .

Tinea corporis occurs most frequently in post-pubertal children and young adults. Rare cases have been reported in the newborn period (Leung *et al* ., 2018) .

1.2.6.2 Tinea unguium (onychomycosis)

Nail infections is a common nail disease, responsible for up to 50% of diseases of the nail. It is characterized by thickened, discolored, broken and dystrophic nails. The nail plate may be separated from the nail bed. Toenails are affected more often than fingernails (Le Grand , 2003).

Two dermatophytes, *Trichophyton rubrum* and *Trichophyton mentagrophytes*, account for more than 90% of onychomycoses. Onychomycosis can be divided into four major clinical presentations: distal subungal (the most common form of the disease), proximal subungal (the most common form found in patients with human immunodeficiency virus infection), and superficial and total dystrophic onychomycosis. Distal subungal infections can be further subdivided into distal lateral subungal onychomycosis and endonyx subunga onychomycosis. Distal lateral subungal infection, the most common form of the disease, begins on the distal section of the nail and spreads under the nail , Endonyx subungal onychomycosis is a subungal form of the disease that involves infection of the nail plate (Le Grand , 2003).

1.2.6.3 Tinea pedis

Tinea pedis is usually caused by dermatophytes such as *T. mentagrophytes* **,** manifests as vesicles, small bullae, and occasionally erosions, particularly on the instep of the plantar surface of the foot and interdigital web spaces. And *T.rubrum* , manifests as peeling, and fissuring of the intertriginous areas of the toes**.** Pruritus is the most prominent symptom (Cynthia and Joseph , 2015).

The skin of the foot represents a unique environment, with a large number of sweat glands but lack of sebaceous glands. Sebaceous secretions are thought to have antimicrobial properties . Furthermore, wearing occlusive shoes may create a high relative humidity and warm conditions. The fungi thrive in warm and humid conditions . Tinea pedis has a high infectivity and recurrence rate, which affects

the quality of life of patients. Characterized of tinea pedis including dryness, fissures and scales or white, moist macerated lesions in some or all of the spaces between the toes. Another form of tinea pedis appears as scaling of the soles and lateral surfaces of the feet, with variable degrees of inflammation and dryness. And other form of tinea pedis is characterized by erythema, vesicles, pustules and bullae mainly on the soles of the feet (Xiaoping *et al* ., 2019) .

1.2.6.4 Tinea cruris

Tinea cruris known as jock itch, is an infection involving the genital, pubic, and perianal skin caused by pathogenic fungi known as *Trichophyton rubrum* , *Trichophyton mentagrophytes* (Khurana *et al* ., 2019)

These *Trichophyton* affect keratinized structures such as hair and the epidermis' stratum corneum resulting in a characteristic rash. Intertriginous areas are hospitable environments for fungus, with sweating, and alkaline pH being responsible for the groin's predilection for infection (Khurana *et al* ., 2019) .

Also, several risk factors have been identified that predispose an individual to tinea cruris, including excessive perspiration, occlusive clothing, improper hygiene, diabetes mellitus,immunocompromise, and lower socioeconomic status (Gupta *et al* ., 2017) .

The symptoms include burning, pruritus, and erythematous lesions with scales, raised, sharply demarcated borders and central clearing. Pustules and vesicles are sometimes found at the edges of the lesion. Macerated, moist exudative forms or lesions with an eczematous appearance can be present in acute cases, while dry lesions with little scaling and an annular form are more characteristic of chronic cases. Hyperpigmentation is common in the central region as the lesion progresses. On the other hand ,The differential diagnosis for tinea cruris includes several other dermatologic conditions affecting the groin with similar presentations. Candidiasis, erythrasma and psoriasis. Unlike tinea cruris,

candidal intertrigo frequently affects women, and the rash may involve the scrotum and penis in males. Satellite lesions and erythema without central clearing are indicative of candida as opposed to tinea. The rash of erythrasma lacks an active border and demonstrates coral-red fluorescence on wood lamp examination (Micah, 2022).

1.2.7 Virulence factors of *Trichophyton species*

The fungal cell wall directly interacts with the external environment and is vital to the survival of the organism. The cell wall supports fungal growth and development and enables the organism to endure hostile ambient conditions (Martins *et al* ., 2019).

Trichophyton species relies on various virulence factors to initiate the Infection. It frequently causes chronic infections of skin, nails and scalp, granulomatous lesions may sometimes occur (de Hoog *et al* ., 2017) .

Fungal virulence is determined by the coordinated expression of various genes mediating host-fungus interactions. Identification of the specific gene products or pathways crucial for fungal survival and infectiveness may potentially direct the development of new antifungal therapeutics.The fungal cell wall is the most promising virulence factor target for drug discovery because of its location, mediation of fungal-host interactions, uniqueness of composition, and absolute necessity for the survival of the pathogen (Maíra *et al* ., 2019) .

The roles of the cell wall in adhesion, colonization, signaling, and immune recognition make it vital for pathogen infection (Hasim, *et al* ., 2019) .

The enzymatic and non-enzymatic virulence factors of the organisms play a major role in these infections. Enzymes secreted by *Trichopytones* seem to be not only virulence factors and indeed not all of them play an overlapping role during infection. Some of the enzymes may be used only during specific stages of infection or might have a more general role in growth that is not specific to

virulence . Furthermore, it seems that each of the *Ttrichopytones* species and, possibly, strains has a unique profile of enzymes and other putative virulence factors during infection (Gnat *et al* ., 2018) .

1.2.7.1 Keratinase

Keratin is important and needed for the growth of *Trichohyton species* in the host tissue.The ability to invade keratinized tissues is defined as a pivotal virulence attribute of this group of medically important fungi. The host–*Trichophyton* interaction is accompanied by an adaptation of fungal metabolism that allows them to adhere to the host tissue as well as utilize the available nutrients necessary for their survival and growth(Mariyammal *et al* ., 2018) .

Keratin can be classified as α-keratin and β-keratin according to the composition of amino acids and the secondary structure of polypeptide chains (Lange *et al* ., 2016) .

It is shown that α-keratin is mainly present in mammals and β-keratin is in avian and reptilian tissues. The polypeptide chains are packed into the final structure through disulfide bonds formed by cysteine residues, hydrogen bonds, and hydrophobic interactions (Vidmar and Vodovnik, 2018).

Cysteine residues play a key role in the structural stability of keratin by forming intra-or intermolecular disulfide bonds (Barone *et al* ., 2005). Keratin is also classified as hard keratin and soft keratin based on the content of cysteine . As basic units of keratin are polypeptides, keratinases play a major role in keratin degradation by breaking the disulfide bonds and peptidic bonds (de Menezes *et al* 2021) .

The range of activity of keratinases from various *Trichohytones* are limited to specific species of animals and groups of people . Keratins are a family of important structural proteins found in nails, skin and hair.keratin exhibits an

elevated content of several amino acids such as glycine, alanine, serine, cysteine and valine.

Because of a high degree of cross-linking by cysteine disulfide bonds, hydrogen bonding, and hydrophobic interactions keratin is insoluble and not degradable by proteases such as trypsin, pepsin, and papain . Keratinase is produced only in the presence of keratin substrate (Mariyammal *et al*.,2018).
Keratinase enzymes from microbes have some distinctive properties with regard to temperature, pH, specificity, activity and stability (Salil, 2019) .

Keratinases are predominantly secreted extracellularly by a diverse range of microorganisms growing in media containing keratinous substrates (Fungi ,bacteria and actinomycetes) .

Fungal keratitis is more common in males than in females. Risk factors are agricultural occupations, age, pre-existing ocular disease, exposure keratopathy, chronic keratitis, chronic use of steroids, diabetes, systemic immunosuppressive disease and Environmental conditions including temperature, annual rainfall, windy seasons and the harvest period have a significant role in increasing the incidence. The incidence of fungal infections is higher in tropical and semitropical areas and is much more frequent in developing countries. In some hot and humid regions it accounts for 50% of cases (Mravičić *et al* ., 2010) .

During growth, keratin is the only source of carbon for *Trichophyton* , and as a result of its breakdown, the pH of the host's skin changes from acidic to alkaline. This adaptive response in turn provides a favourable pH for optimal activity of most proteases secreted by *Trichoytones* and is essential for survival, virulence and dissemination in various host niches , that the metabolism of some amino acids, i.e. glycine released from skin proteins, result in the secretion of ammonia and a change in the pH in media from acidic to alkaline , During the alkalinization process *Trichopytone* respond by expressing enzymes that are functional at an

acidic or alkaline pH, depending on the prevailing condition (Sebastian Gnat *et al* ., 2019).

1.2.7.2 Phospholipases

Phospholipases, as one of the virulence markers, facilitate the colonization of host cells by attaching the *Trichopyton* to the target tissue and destroying the cell membrane following the hydrolysis of phospholipids in host tissues, releasing fatty acids and lysophospholipids that can be used as a nutrient source by the fungus , confirm the importance of this enzyme in the main stage of *Trichophyton* infection and fungal colonization , these enzymes may play an important role in balancing host immunity and the ability of *Trichophyton* to reduce the immune response(Anita *et al* ., 2021).

Phospholipases enhance virulence by damaging host cell membranes , these hydrolytic enzymes may have other functions that facilitate virulence, in addition to causing direct tissue damage .

The phospholipases are heterogeneous group of enzymes which are capable of hydrolysing one or more ester linkage glycerophospholipids. Phospholipase activity is responsible for the hydrolysis of easter linkage and destabilizes the cell membranes of the host. Phospholipases are categorized into five groups depending on the ester bond hydrolysed: A1, A2, B, C, and D (Zuzarte *et al* ., 2021) **.**

Phospholipases have been proposed as potential targets for antifungal therapies, as inhibiting their activity may reduce the virulence of dermatophytes and improve the efficacy of antifungal agents (Baeza *et al* .,2017) .

1.2.7.3 Transcription factor PacC

During infection, a complex and orchestrated circuit of intracellular signaling is activated to regulate responsive genes involved in the adherence, penetration, and maintenance of *Trichopyton* in the host environment . The first interaction of *Trichopyton* with skin and nails occurs under acidic pH conditions. The

maintenance of skin pH at an average of 4.7, is related to defense against infections . It is promoted by combining molecules, such as acid lipids, amino acids, free fatty acids from glands and epidermal cells, and resident microbiota (Martinez *et al* ., 2017) .

The signaling pathways that transduce the extracellular pH and allow a prompt response to these changes are relevant for adaptation, survival, growth, dissemination in different niches, and virulence . The transcription factor PacC, a well-known pH response pathway component, plays a role in pathogenesis and immune modulation during fungal infection . In dermatophytes, PacC plays a role in virulence, including protease secretion, keratinolytic activity, and growth in human (Ferreira *et al* ., 2006).

The PacC signaling pathway comprises six proteins, PalH, PalI, PalF, PalC, PalA, and PalB, which convey any change in environmental pH to the transcription factor PacC, which commences at neutral to alkaline pH . the full-length PacC protein would be inactive under acidic conditions. PacC is involved in mannosyltransferase regulation, and transcription factors are also related to HSP regulation (Nilce *et al* ., 2021) .

The *pacC* gene is required for the import and export of essential substrates, to control ion homeostasis (Anita *et al* ., 2021) and to participate in the direct regulation of salt tolerance by controlling efflux systems. It also interferes with the modulation of efflux-associated genes in a time-dependent manner (Lanza *et al* ., 2019) .

1.2.7.4 Heat Shock Protein (HSP90)

Most of the virulence factors are under the restrict control of regulatory elements such as heat shock proteins (*Hsps*). Hsp90 is molecular chaperone involved in the regulation of cellular signaling in eukaryotes. HSP90 is a

ubiquitously expressed chaperone involved in the proper folding of various protein kinases, nuclear receptors, and transcription factors. HSP90 plays crucial roles in the maintenance of cell homeostasis. It has an inevitable role in pathogenesis and life cycle of *Trichophytone* . Considering the crucial role of HSP90, any modification in expression pattern of the protein has a vast effect on viability, morphology, and pathogenicity of the organism (Kumawat *et al* ., 2022) .

Hsp90 governs cellular morphogenesis, drug resistance, and virulence . Treatment of fungal infections is difficult due to several reasons, such as side effects of drugs, emergence of resistant strains, and limited number of molecular targets for the drug compounds. In fungi, heat shock proteins (Hsps) have been implicated in several processes with the conserved molecular chaperone Hsp90 emerging as a potential target for antifungal therapy. It plays key cellular roles by eliciting molecular response to environmental changes, morphogenesis, antifungal resistance, and fungal pathogenicity (Tiago *et al* ., 2015) .

This protein rapidly accumulate in the cytosol in response to heat and environmental challenges such as antifungal drugs, oxidative stress, and heavy metal exposure among others. The heat shock response (HSR) is considered a rescue mechanism that enables the cells to cope under stressful conditions and protects from severe damage. The primary role of Hsps is to sense and assist proper protein folding and refolding, and direct them for degradation in case of misfolding, thereby assuring proteome integrity and homeostasis (Tiago *et al* ., 2015) .

Hsps act as molecular chaperones or transcriptional regulators in a myriad of physiological functions. In *Trichopyton* spp , Hsps have been implicated in several processes, including pathogenicity, phase transition in dimorphic fungi, and antifungal drug resistance(Brown *et al*., 2010).

However , Hsps are synthesized as an adaptive response to stress that contributes to the survival of pathogenic microorganisms in the host. Hsp90 can associate with several proteins involved in signaling, metabolism, cell growth, transcription, protein trafficking, chromatin remodeling, and stress response, among others. The energy produced by the hydrolysis of ATP is used by Hsp90 to fold the target proteins to their active conformations (Tiago *et al* ., 2015) .

1.2.8 Multilocus sequence typing (MLST)

Multilocus sequence typing is a identical technique has been used by mycologists to study basic evolutionary features of human pathogenic Fungi. MLST has become popular because it samples the tremendous polymorphism present in nucleotide sequences and because each new study can use and add to all obtained data (Morehouse *et al* ., 2003). It is a very stable and reproducible technique that determines the isolates of the microbial species using the DNA sequences of multiple housekeeping genes (Bernhardt *et al* ., 2013) .

MLST, which is a typing method based on the sequencing of housekeeping genes and characterizes isolates on the basis of variation in nucleotide sequences of each locus of the selected genes. The different sequence at each locus are assigned with specific allele numbers and each unique combination of alleles, often called as allelic profile is assigned a sequence type (ST), which is the unambiguous descriptor of the strain (Zhang *et al* ., 2018) .

MLST results are unequivocal, and sequence data can be shared and compared between different laboratories. More specifically, the multilocus sequence can distinguish between the individual strains and other isolates, and evaluate the relationships, recombination, and mutation rates among microorganisms, which are classified under the same genus (Bougnoux *et al* ., 2002) .

The phylogenetic species concepts and phylogenetic analysis based on the internal transcribed spacer (ITS) regions contribute to the improvement of the taxonomy. At present, the sequence database of the ITS region is considered to be the gold standard for dermatophytes. Sequencing of the *Beta-tubulin (BT2), translation elongation factor 1-a (TEF-1a) genes, calmodulin (CaM), or sequencing the D1/D2* domain of the large-subunit
rRNA gene, as well as the ITS region, can reliably identify the species (Ahmadi *et al* ., 2016) .

MLST also has the advantage over single nucleotide polymorphism (SNP) analysis that new polymorphic nucleotide positions in any of the gene fragment sequence can be detected and added to the database. This feature makes it possible to add new individuals from new geographic locations to the study without the danger that variation found to be polymorphic in the
initial population will be monomorphic in the newly added ones, as can happen with SNPs (Burt *et al* ., 1997) .

MLST can be used to help discriminate among the several possible causes of an emerging disease. For example, the fungus may have been in the area all along, and is now causing disease because the host has become susceptible or the pathogen has become more prevalent. In this case, MLST of fungal individuals causing disease should be no different than individuals obtained from the environment, and all fungal individuals in the area of disease outbreak should show population structure similar to that found in other geographic locations .The ability to accurately distinguish between strains of infectious pathogens is crucial for efficient epidemiological and surveillance analysis, studying microbial population structure and dynamics and, ultimately, developing improved public health control strategies. (Eduardo and Miguel 2017).

Since 1998, the established standard for molecular typing is multilocus sequence typing (MLST). MLST was built on the well-established population genetic concepts and methods of the multilocus enzyme electrophoresis (MLEE) technique, but provides significant advantages over this and other typing approaches. MLST examines nucleotide variation in sequences of internal fragments of usually seven housekeeping genes: that is, those encoding fundamental metabolic functions. Also The principal element in the design of an MLST scheme is the choice of genetic loci. (Eduardo and Miguel ,2017) .

The development of a new MLST scheme from involves four initial steps (1) identification of loci, (2) PCR primer design, (3) survey of a small number of representative strains, and (4) analysis of nucleotide sequence data to establish neutral evolution of loci and level of strain discrimination.

At least 7- 9 loci that could be amplified from all test strains and showed a reasonably high level of genetic diversity. MLST targets variation at multiple housekeeping loci (Pérez *et al* ., 2013).

MLST has become the most commonly used method of pathogen typing. In comparison to older methods , the use of genetic variation gives MLST the advantage of producing variable data (more resolution) that are universally comparable (within schemes), easily validated, and readily shared across laboratories. The use of generic sequencing technology makes MLST a broadly applicable methodology that can be fully automated and scalable from single isolates to thousands of samples. Because the materials needed for MLST analysis DNA or dead cells are easily transported among laboratories without the problems associated with infective materials, both the biological samples and the resulting data are highly portable.(Jefferies *et al* ., 2003) .

By focusing on sequence variation, MLST provides a highly replicable and reproducible typing method. Additionally, the focus on housekeeping genes

provides significant amounts of genetic data that can be used to calculate pathogen population genetic parameters. Those parameters can be then used to construct more sophisticated models of pathogen evolution and epidemiology that will improve understanding of how to control the spread of disease(Pérez *et al* ., 2013).

In MLST the number of nucleotide differences between alleles is ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites. The rationale is that a single genetic event resulting in a new allele can occur by a point mutation (altering only a single nucleotide site), or by a recombinational replacement (that will often change multiple sites) - weighting according to the number of nucleotide differences between alleles would erroneously consider the allele to be more different than by treating the nucleotide changes as a single genetic event .

In a typical MLST approach, recombination is expected to occur with a much higher frequency than point mutations. Therefore, one does not look at the total sequence similarity between strains. Instead, each sequence for a given locus is screened for identity with already known sequences for that locus. If the sequence is different, it is considered to be a new allele and is assigned a unique (arbitrary) allele number (Martin , 2006) .

1.2.9 Whole genome sequence (WGS)

Since the discovery of DNA structure by Watson and Crick (Watson and Crick, 1953). Several molecular approaches were developed to detect the sequence of DNA bases. The pioneering development of these approaches facilitates evolution of the field of DNA sequencing in applications, capacity and capability (Levy and Myers, 2016). Whole genome sequencing (WGS) represents one of these methods that determine DNA bases at the genome scale. WGS provides the most comprehensive identification of genetic variation for any organism .

In addition, sequencing multiple genomes provides important insights about the genetic variation and polymorphisms among their genomes that in turn involved in identification of nonsynonymous or synonymous mutations (Sjoblom *et al*., 2006) .

In addition, WGS-based identification of virulence, antifungal resistance, and toxin genes could be more suitable and successful than PCR-based identification methods during outbreak circumstances, where this high resolution identification of pathogenicity genes might lead improved virulence characterization and outbreak control(Köser *et al* ,. 2012) **.**

WGS can also be used to identify the path of disease transmission within a population and provide information on the probable source. It is essential for mutation detection and in understanding of genetics of microorganisms. It can also evaluate the evolution of strains during an outbreak and detect contextual data on the genetic interrelatedness (Gilchrist *et al*., 2015) .

Whole genome sequencing can be used to identify pathogenic fungi, genes related to pathogen and host interaction, and the evolutionary relationships among closely related species (Chitra *et al* ., 2015)**.**

For edible and medicinal fungi, it can be used to discover complex metabolic pathways and to identify metabolites which are beneficial to human health. And some fungi have implications for biological control or industrial applications .Bioinformatics analysis includes: genomic assembly, genomic structure studies, functional annotation, pathogenic microbiology, and comparative genomic analysis (Martinez *et al* ,. 2004) .

The availability of these sequence and analysis data will provide researchers large amounts of useful information that will provide power to studies aimed to decipher and interpret the molecular basis of host colonization, invasion, and specialization next generation sequencing (NGS) and long-read sequencing

technologies to sequence the fungal whole genome. Fungal whole genome sequencing includes two categories: fungal genome de novo sequencing and fungal genome resequencing. The workflow for fungal genome de novo sequencing includes the following steps: (1) the chromosomal DNA is randomly interrupted; (2) different lengths of sequences are selected to construct a library; (3) large-scale sequencing; (4) genomic assembly without any prior genome information; (5) fill the gaps on the draft genomes (Quail *et al* ., 2012) .

The genome sequence of fungal can also be determined through alignment to reference genome sequences using the homogeny of sequences as an index. It is a quick and accurate method for detection of variants. the fungal genome sequence, helping to improve medical science, agriculture science, ecology, bioremediation, bioenergy, and biotech industries (Quail *et al* ., 2012) .

Given the major progress in the development of broad-scale transcriptional and genome sequence-dependent analyses of dermatophytes .

These genome analyses identify gene families that are important to understanding of how dermatophytes cause chronic infections, how they interact with epithelial cells, and how they respond to the host immune response. These genome sequences provide a strong foundation for future work in understanding how dermatophytes cause disease (Burmester *et al*., 2011) .

Genomic studies of human pathogenic fungal species have revealed the repertoire of proteins that contribute to host interactions by providing a comprehensive picture of gene content and functional potential . High-resolution whole genome sequencing promises to revolutionize surveillance and diagnosis of infectious diseases . Not only does the genome of provide genetic resources for comparative genomics, evolutionary studies, and alleviating the bottlenecks in molecular breeding like in edible mushroom (Li *et al* ., 2022) .

The final determinant of characteristics such virulent as well as antifungal resistance is genetic material. In order to identify the genetic components of these features, it appears to be promising to analyze genome sequences of species or isolates with different virulence or resistance profiles. The genomes of fungal pathogens are really quite dynamic, with significant differences across and within species (Tamarit *et al* ., 2022) .

Generally, there are presently two major approaches performing WGS, First generation sequencing (e.g., Sanger sequencing) and Next generation sequencing (NGS). Therefore, this technology is widely used for infection control and prevention processes in the clinical microbiology laboratories Regarding NGS technology, there are presently two major technologies: 1- Short read sequencing technologies (a.k.a Second generation sequencing) 2- long read technologies (a.k.a Third generation sequencing).

1.2.9.1 Next Generation Sequencing (NGS)

Next-generation sequencing systems have made substantial advances in DNA sequencing technologies, providing higher accuracy and considerably lower costs (Liu *et al*., 2012). The number of whole genomes stored in public repositories, such as the Online Genome Database (https://gold.jgi.doe.gov), has grown exponentially due to next-generation technologies. Comparative analyses, such as pan-genomic analysis, have become possible with the great number of genomes available, particularly the prokaryotic genomes which drive gene discovery in the biomedical, biotechnological, and environmental fields. Comparative genomics was used to examine intrinsic genomic features in other species. The virulence mechanisms in pathogenic organisms may be elucidated through the pan-genomic method by using multiple organisms of a single species or genera for the identification of similarities between genomes . This approach

can map the occurrence of and establish phylogenetic relationships for evolutionary events (Metzker, 2010).

Furthermore, comparative genomics can be employed in microorganisms with different habits to compare their gene repertoires and genome sizes as intracellular pathogens often encounter reduced evolution and gene loss (De Veerdonk *et al* ., 2017) .

In comparison with the traditional Sanger method, the NGS technologies have enhanced the sequencing and analysis speed, and they provide massively high-throughput from multiple samples as well as reduced (Mardis *et al* , 2011).

In addition to DNA sequencing, NGS technologies can be used for RNA sequencing (a.k.a where whole transcriptome sequencing, WTS), methylation sequencing (MeS), whole-exome sequencing (WES) and Candidate gene sequencing (CGS) (Kulski, 2016)**.**

Regarding NGS technology, there are presently two major technologies: 1- Short read sequencing technologies (a.k.a Second generation sequencing) 2- long read technologies (a.k.a Third generation sequencing) .

1.2.9.1.1 Second Generation Sequencing

Second generation sequencing approach provides massively parallel sequencing with a reduced cost and higher accuracy data (Goodwin *et al*., 2016). These approaches are emerged as a typical tool for clinical use in population level studies and in variant discovery for surveillance of infectious disease (Ardui *et al*., 2018) . The core principle of second-generation sequencing technology is mainly depending on chemical reaction and optical detection processes (Suwinski *et al*., 2019).

This technology performs parallel sequencing of random fragmented pieces of genomic DNA or cDNA without cloning step via a foreign host cell that is needed in first generation sequencing. In contrast to first generation sequencing, the

fragmented pieces of DNA or cDNA are ligated to linker or adapter sequences to construct template libraries. These libraries amplified on a solid surface or on beads and then insulated within arrays or tiny emulsion droplets and Nucleotide identification is performed by luminescence detection or by electrical charge detection. They generate billions of nucleotide sequences within each single run, where very large datasets are produced after sequencing each genome in multiple times (Besser *et al*., 2018).

Second generation sequencing technology has different commercial platforms that differ markedly in terms of their chemical reaction, output length, accuracy and cost (Buermans and Den Dunnen, 2014). These technologies included: (1) pyrosequencing (454/Roche), this technology depending on pyrosequencing immobilize DNA on beads and computationally detection of pyrophosphate molecule that is enzymatically released after incorporation an un-labelled nucleotide into the newly synthesized strand chain(Mardis, 2008) .(2)Sequencing by Oligonucleotide Ligation and Detection (SOLiD/Life Technologies), it is based mainly on nucleotide sequencing by ligation of a fluorescently labeled DNA with the universal primer to identify the nucleotide position in each sequencing cycle (Margulies *et al*., 2005) , (3) semiconductor sequencing (Ion Torrent), this technology rely on the electronically detection of the nucleotide sequences inside microchip by detection of the pH changes after incorporate new nucleotide (Wang *et al*., 2014), (4) Sequencing by synthesis Illumina (SOLEXA), Illumina technology is based on sequencing by synthesis of the complementary strand to the adaptor attached on a glass solid phase surface and detection of terminator nucleotides by fluorescence(Bentley *et al*., 2008).

The Illumina platform represents the most successful sequencing system and currently occupies a major part of NGS technologies with more than 70% dominance of NGS market . Industrially, Illumina provides multiple instruments:

MiniSeq, MiSeq, HiSeq, NextSeq and NovaSeq, where they are varied with throughput and read lengths (Bharagava *et al*., 2019). The MiniSeq and MiSeq machines generate low to mid throughput for each sample, inexpensive instrument prices, and are represent a reasonable choice for small public health laboratories, whilst, the extremely expensive HiSeq, NextSeq and NovaSeq instruments generate much higher throughput and reduced the per sample cost, and are therefore more suitable for large sequencing facilities . However, All Illumina platforms have commonly been used for investigating of pathogens during infectious disease outbreaks (Raghavendra and Pullaiah, 2018).

The workflow of Illumina platform includes several steps(1) extraction of genomic DNA from sample; (2) Preparation of library, which ordinarily involves fragmentation of genomic DNA randomly and the ligation of adaptors;(3) Amplification of DNA fragments by Bridge PCR strategy, where this method amplifies the DNA fragment that have adaptors attached to complementary primers on the glass surface of the flowcell, this method generates a cluster of amplicons; (4) Automated sequencing and fluorescently detection of reversible-terminating nucleotides, as shown in Figure (1-1) (Buermans and Den Dunnen, 2014).

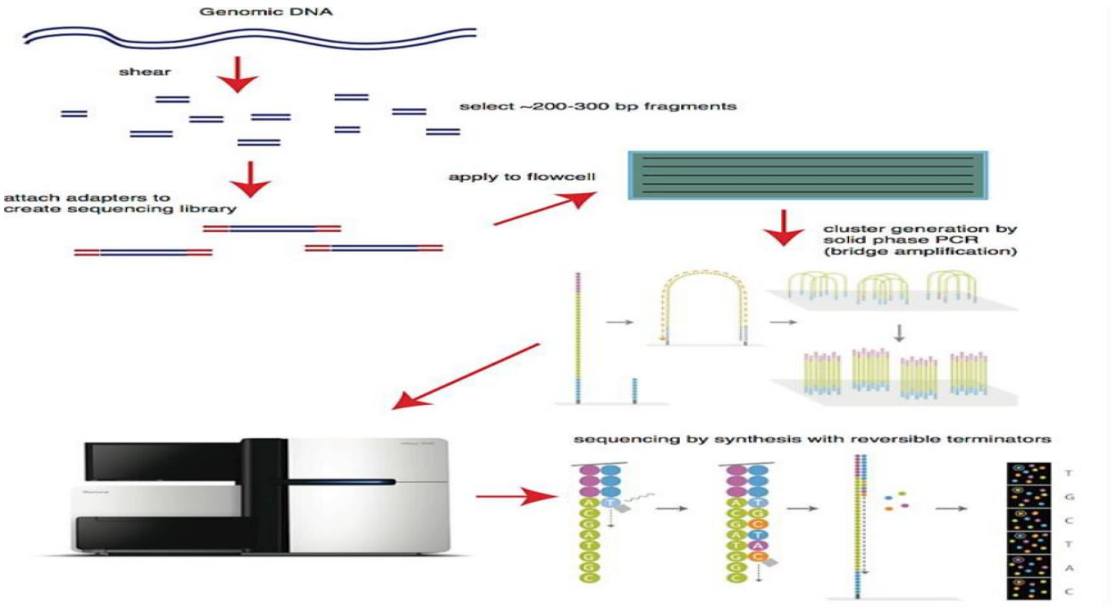


Fig. (1-1): An overview of Illumina sequencing technology

1.2.9.1.2 Third Generation Sequencing

Third-generation sequencing technologies have designed to address several limitations emerged in second-generation sequencing technologies such as PCR amplification of the templates step, where this technology is characterized by sequencing of only one DNA molecule, i.e., the optic sensors are highly sensitive for fluorescently based detection of one nucleotide insertion (Méndez-Vilas and Teixeira, 2010). Directly sequencing of single DNA molecules in these technologies reduced low error rates, GC bias, intensity averaging, phasing alleles problems(Eid *et al*., 2009; Schadt *et al*., 2010). Even though third-generation sequencing technologies offer high accuracies, detection of epigenetic modifications, isoforms discovery and in applications of de novo genome assembly, they still have limited utility in the clinical setting compared with Short read sequencing technologies due to the low throughput and expensive cost(Goodwin *et al*.,2016) .

Commercially, there are three providers for single molecule long read sequencing technology varying in their strategies: 1- Single-molecule realtime

sequencing (SMRT) by Pacific Biosciences, which it uses fluorescent nucleotides introduced into the reaction wells of their SMRT cell; 2- Nanopore sequencing (MinION) by Oxford Nanopore Technologies, these platforms guided DNA into nano-pores and detection of type of nucleotide is occurs by using ionic current sensing; 3- HeliScope sequencing system by Helicos BioSciences, this system performs single DNA molecules sequencing by synthesis and detects nucleotides that incorporated into the growing strand by using a highly sensitive fluorescence image system (Rhoads and Au, 2015) .

2.1. Materials and Methods

2.1.1 Specimens

This study includes 150 specimens (patients) which were collected from skin and nail. The Patients were visited to dermatology outpatient clinic of Marjan teaching hospital , and dermatology outpatient clinic Al‑Sadeq teaching hospital , these specimens diagnostic was done clinically and Full information were detected from each patient including : age ,sex ,clinical type and severity by dermatologist between April and October 2022 . The patient's age ranged from (17 years - 65 years).

2.1.2. Laboratory Equipments and Instruments

The main equipments and instruments used throughout this study are listed in table (2-1).

Table (2-1) Laboratory Equipments and Instruments

No.	*Instruments*	*Company*	*Country*
1.	Incubator	Memmert	Germany
2.	Water bath		
3.	Distillator	GFL	Germany
4.	Centrifuge	Hettich	
5.	PCR tubes	Eppendorf	
6.	DNA extraction tubes.		
7.	Micropipettes 5-50 µl ,100-1000 µl, 0.5 – 10 µl		
8.	pH-meter	Gallenkamp	England
9.	Platinum wire loop	Himedia	India
10.	A autoclave	Stermite	

11	Light microscope	Olympus	Japan
12.	Sensitive electron balance	A & D	
13.	Dualed Blue /White Transilluminator and documentation system	Bioneer	Korea
14.	Wooden sticks	Supreme	China
15.	Refrigerator	Concord	Italy.
16.	Plastic Test tubes 10ml.	AFCO	Jordan
17.	PCR system	Cleaver	USA
18	Greenstar	Bioneer	Korea
19.	Gel electrophoresis	Cleaver	USA
20.	Vortex	Germmy	Twain
21.	Hood	Labogene	Danemark
22.	Parafilm	Bemis	USA
23 .	Tips	Dolphin	Syria
24	Medical gloves	Broche	China
25 .	Pettri dishes	Blastilab	Lebanon
26.	Medical cotton	Medicare Hygiene Limited	India
27 .	Glass slides	Sail brand	China
28 .	Microscopic Cover slide	Gitoglas	China

2.1.3. Chemical and Biological Materials

2.1.3.1. Chemical Materials

Table (2-2) Chemical Materials

N0.	*Chemicals*	*Company/country*
1.	Lactophenol-cotton blue	CDH / India
2.	KOH 10%	Searle/England
3.	Alcohol (Ethanol)70%	Fluka / Switzerland
4.	Greenstar, Loading dye (bromophenole blue), Agarose, Master mix	(Promega, USA)
5	Tris-Borate-EDTA (TBE) buffer	Bio Basic/ Canada

2.1.3.2. Biological Materials

Table (2-3) Culture Media

No.	Media	Company/country
1.	Sabouraud Dextrose Agar	Himedia , India
2	Sabouraud Dextrose broth	Himedia , India
3	Potato Dextrose Agar	Himedia , India
4	BCP-Milk Solids-Glucose	Himedia , India
5	dermatophyte Test Medium	Himedia , India
6	Corn meal agar	Himedia , India

2.1.3.3 Antibiotics

Table (2-4) Antibiotics

Antibiotics	*Company*	country
Cychloheximide	HI Media	India
Chloramphenicol	BDH	England
Gentamycin	BDH	England

2.1.4 Commercial kits

Table (2-5) Commercial kits used in the present study

No.	*Type of kits*	*Company/country*
1.	DNA extraction kit	Favogene /Taiwan
2.	Green master mix Kit	Promega /USA
3.	DNA ladder	Promega /USA

DNA extraction kit
Materials: Proteinase K , TG 1,2 Buffer , Ethanol (98 -100 %) , 2 ml Collection Tube, Wash Buffer, Elution Buffer , RNase , FB Buffer .
Green master mix Kit

Materials:

1- DNA polymerase enzyme (Taq).

2- dNTPs (400µm dATP, 400µm d GTP, 400µm dCTP, 400µm dTTP)

3-Reaction buffer (pH 8.3)

DNA ladder

Materials:

1-Ladder consist of 13 double-stranded DNA ranging in size from 100 to 2000 size bp .

2-Loading Dye which has a composition of (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol,.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA)

2.2. Methods

2.2.1 Sterilization Methods

- Sterilization of the culture media , buffer and solution by autoclave at 121ºC and pressure 15 psi for 15 minute.

2.2.2 Preparation of Solutions and Reagents

2.2.2.1. Normal Saline Solution

It was prepared by dissolving 8.5 gm of NaCl in a small volume of distilled water, then completed to 1000 ml, pH fixed at 7.2 and sterilized in autoclave (MacFadden, 2000).

2.2.2.2 Potassium hydroxide solution (10% KOH)

It was prepared by dissolving 10 g of potassium hydroxide in 100 ml of distilled water. 10% potassium hydroxide solution was used to observe the fungal structures upon direct microscopic examination for clinical models (Meija *et al*.,216) .

2.2.3 Preparation of Culture Media

All media were prepared according to the manufactures instructions fixed on their containers, they were sterilized by autoclave at 15 psi/inch in 121ºC for 15 min , 20 ml of sterilized media were poured in disposable Petri dishes and they were incubated at 30 C ºfor overnight to ensure sterility, and stored at 4ºC until used.

2.2.3.1 Sabouraud Dextrose Agar (SDA) Medium

SDA medium was prepared according to the manufacturer's instructions, by dissolved 64 gm of SDA in 1000 ml of distilled water, then sterilized by autoclave, this medium was used for culturing and sub culturing the dermatophytes isolates (De Hoog *et al* ., 2009) .

2.2.3.2 Potato Dextrose Agar (PDA) Medium

PDA medium was prepared according to the manufactures instructions, by dissolving 39 gm of PDA in 1000 ml of distilled water, sterilized and poured in sterilized plates. (Atlas , R.M 2010).

2.2.3.3 Bromocresol purple (BCP)Milk Solids-Glucose Medium

This medium is available commercially as Dermatophyte Milk Agar (Hardy). Type of growth and a change in the pH indicator (BCP) indicating alkalinity are especially useful for distinguishing *T. rubrum* from the *T. mentagrophytes* complex. *T. rubrum* shows restricted growth and produces no alkaline reaction on

BCPMSG , whereas members of the *T* . *mentagrophytes* complex typically show profuse growth and an alkaline reaction (De La *et al* ., 2018) .

2.2.3.4 dermatophyte Test Medium

It is a specialized agar medium used in the laboratory to cultivate and identify dermatophyte fungi**.** DTM contains a pH indicator that changes color in the presence of dermatophytes, allowing for easy identification of fungal growth. *Trichophyton rubrum* colonies on DTM appear red or pink, and grow more slowly than *Trichophyton mentagrophytes*, which produces a more rapid and dense growth and may appear yellow or orange on DTM (El-Khalawany *et al* ., 2021).

2.2.3.5 Corn meal agar

It is a type of agar medium used in the laboratory to cultivate and identify fungi, including dermatophytes. It contains cornmeal as a nutrient source and can be used to observe the growth patterns and morphological characteristics of fungal colonies. *Trichophyton rubrum* typically grows as a fluffy, white colony with a pink center, while *Trichophyton mentagrophytes* grows as a more granular, yellowish-white colony (Sachdeva *et al* ., 2021) .

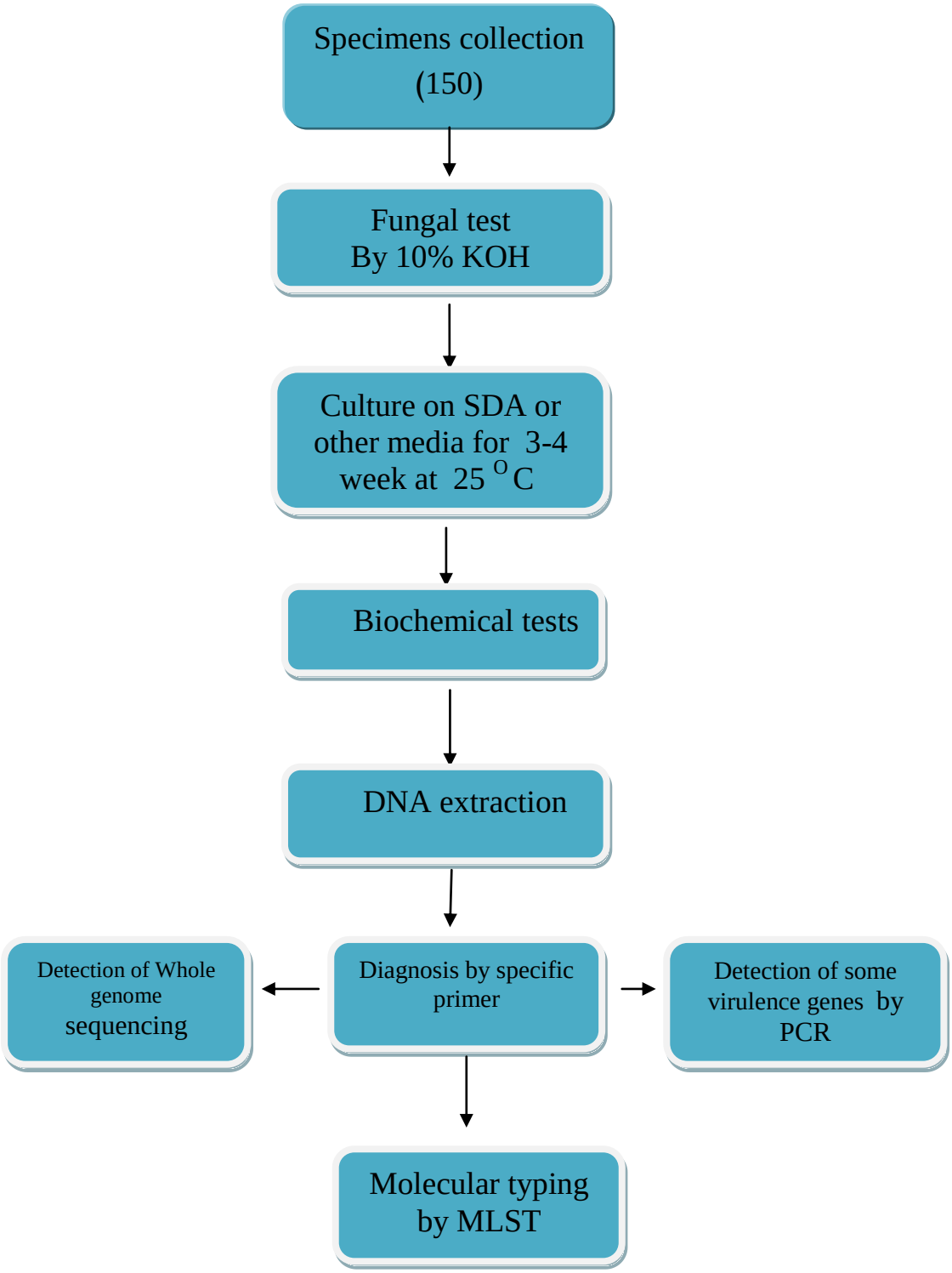
2.2.3.6 Agarose Gel

Agarose gel was prepared by dissolving 0.6 gm agarose in 5 ml TBE buffer, then the volume was completed to 50 ml by deionized water, after boiling, it was left to cool to 50ºC, 0.1 greenstar was added, mixed well and poured to the tray of gel electrophoresis (Sambrook and Russell, 2001).

2.2.4 Subjects of the Study

This study includes 150 specimens (patients) which were collected from skin and nail. The Patients were visited to dermatology outpatient clinic of Marjan teaching hospital , and dermatology outpatient clinic Al‑Sadeq teaching hospital , these specimens diagnostic was done clinically by dermatologists between April and October 2022.

2.2.4.1 Study Design



2.2.4.2 Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. Verbal consent was taken from each patient before sampling. Investigative standards were rigidly preserved, primarily concerning confidentiality. Moreover, this study was undisclosed, participation of patients was optional, and verbal consent was received before data uptake process was started. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee (at College of Medicine University of Babylon) under the reference No BMS/0235/016.

2.2.5 Isolation and identification of *Trichophyton SPP.*

The proper specimens collected for fungal analysis are described below. Those specimens were collected in proper ways to avoid any possible contamination .

2.2.5.1 Collection of skin scrapings and nails for microscopic exam and fungal culture

A. Select affected skin, and nails for laboratory evaluation

B. Collection

1 -Materials

a . Forceps

b. Scalpel

c. 70% alcohol for disinfection

d. Sterile plastic tube or container, envelope

e. Gauze

f. Wood's lamp

2 -Method

a. Skin. Clean the skin surface with 70% alcohol. Scrape the surface of the skin at the active margin of the lesion and remove superficial material. Do not mix blood when scraping the skin Place the scraping in a clean envelope, sterile plastic tube or Container.

b. Nails. Remove nail polish if present from the nail to be sampled Wipe the nail with 70% alcohol on gauze (not cotton). Collect debris from the nail and place it in a clean envelope or plastic tube. Scrape the outer surface of the nail and discard the scraping. Collect scrapings from the deeper, diseased areas of the nail and add them to the material previously collected from under the nail .

C. Label the specimen with patient information.

D. Transport – Do not refrigerate the specimen. Transport at room temperature (Miller,.*et al* 1999).

2.2.5.2 Exclusion criteria

More than twenty cases are excluded from the study due to the absence of study criteria when patient use antifungal after taken the history of patients.

2.2.5.3 Staining

2.2.5.3.1 Lactophenol-cotton blue

It was used to stain and immobilize the fungi for the purpose of the examination microscopy **.**

2.2.5.4 Colonial Morphology and Microscopic Examination

A single colony was taken from each primary positive culture. Its identification depended on the morphology properties (shape, surfue pigmentation , reverse pigmentation , edge, appearance of colonies and elevation of texture) . Texture of the surface (powdery, granular, woolly, cottony, velvety, or glabrous . Topography (elevation, folding, margins, etc.), and rate of growth .The colonies were then investigated by Lactophenol-cotton blue to observe fungal cells.

Microscopic morphology, especially the appearance and arrangement of the conidia (macroconidia or microconidia) and other structures

2.2.5.5 Biochemical Tests

2.2.5.5.1 Hair Perforation Test

In this test, the fungal culture is inoculated onto a hair shaft, and the ability of the fungus to penetrate and digest the hair is observed.

2.2.5.5.2 Urea Hydrolysis

The urease test is commonly performed using culture media containing urea and a pH indicator. The presence of ammonia, resulting from the hydrolysis of urea by urease-positive fungi, leads to an increase in pH, which can be detected by a change in color of the pH indicator

2.2.5.5.3 Nitrate reduction test

The nitrate reduction test is a diagnostic test used in the laboratory to differentiate between different species of *Trihophyton*. In this test, the fungal culture is inoculated onto a nitrate-containing medium, and the ability of the fungus to reduce nitrate to nitrite is observed.

2.2.5.5.4 The Tween 80 hydrolysis

It is a biochemical test that can be used to differentiate between *Trichophyton* based on their ability to produce the enzyme lipase. Lipase is an enzyme that can break down lipids such as Tween 80, a type of fatty acid**.** A fungal isolate is inoculated onto a culture medium containing Tween 80 as the sole source of lipid. After incubation, the medium is examined for evidence of Tween 80 hydrolysis, which is indicated by the formation of a clear zone around the fungal colony.

2.2.5.5.5 Acid production test

The fungal isolate is inoculated onto a culture medium containing a carbohydrate such as glucose or lactose. After incubation, the medium is examined for evidence of acid production, which is indicated by a change in pH.

Trichophyton rubrum is known to be a weak acid producer and may not produce significant amounts of acid on carbohydrate-containing media.

2.2.5.5.6 Indole production test

The indole test is commonly performed by inoculating *Trichophyton* isolates on media supplemented with tryptophan and observing for the production of indole. Indole can be detected using various methods, such as the Kovac's reagent test, which produces a color change in the presence of indole.

Trichophyton based on their ability to produce the enzyme tryptophanase, which can convert the amino acid tryptophan into indole.

2.2.6 Molecular study

2.2.6.1 DNA Extraction

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company (favogen)

1. The sample is grown on Sabouraud's Dextrose Agar.

2. 1~ 5 x10 of cultures (fungal/ cells) to a 1.5 ml microcentrifuge tube .

3. Descend the cells by centrifuging at 5,000 x g for 2 min and discard the supernatant completely.

4. The cells were suspended in 550 µl of FB buffer and was incubated in 50 µl of lyticase solution, mix well by vortexing. Incubat the sample at 37 °C for 30 min.

5.Added 8 µl of 50 mg/ml RNase A (not provided) and incubate for 2 min at room temperature.

6. The cells were descended by centrifuging at 5,000 x g for 10 min. Remove the supernatant completely .

7.Added 350 µl TG1 Buffer and mix well by pipetting. Transfer the sample mixture to a bead tube.

8.Mix well by Plus-vortexing for 5 minutes.

9.Added 20 µl of Proteinase K (10 mg/ml) and mix well by vortexing. Incubate at 55 °C for 15 min, vortex 30 seconds for every 5 minutes incubation.

10. The cells were descended by centrifuging at 5,000 x g for 1 min and transfer 200 µl of supernatant to a new 1.5 ml microcentrifuge tube.

11. Added 200 µl of TG2 Buffer and mix well by pipetting

12. Add 200 µl of ethanol (96-100%) and mix well by pulse-vortexing for 10 seconds.

13. A TG Mini Column was placed in Collection Tube. Transfer the sample mixture (including any precipitate) carefully to TG Mini Column. Centrifuge at 11,000 x g for 30 second then place the TG Mini Column to a new Collection Tube.

14. Added 400 µl of W1 Buffer to the TG Mini Column. Centrifuge at 11,000 x g for 30 seconds and discard the flow-through. Place the TG Mini Column back to the Collection Tube.

15 . Added 750 µl of Wash Buffer to the TG Mini Column. Centrifuge at 11,000 x g for 30 seconds and discard the flow-through. Place the TG Mini Column back to the Collection Tube .

16 . Centrifuge at full speed (~ 18,000 x g) for an additional 3 min to dry the column Important Step.

17 . TG Mini Column was placed to a Elution Tube .

18 . Added 50 ~100 µl of Elution Buffer or ddH2O to the membrane center of the TG Mini Column .

19. Centrifuge at full speed (~ 18,000 x g) for 1 min to elute total DNA.

20. Total DNA was stored at -20°C.

2.2.6.2 Estimation of DNA Concentration

The extracted genomic DNA is checked by using Nanodrop spectrophotometer which measures DNA concentration (ng/µl) and checks the DNA purity by reading the absorbance at (260 /280 nm). As following steps

1- After opening up the Nano drop software, chosen the appropriate application (DNA).

2- A dry paper-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 2µl of ddH2O onto the surface of the lower measurement pedestals for blank the system.

3- The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of extracted DNA carefully pipet onto the surface of the lower measurement pedestals ,then check the concentration and purity of extracted DNA.

2.2.6.3 Dissolving and Preparation of Primers

All primer pairs used in this study were dissolved using TE Buffer, 1X (pH 8.0) composed of 10mM Tris-HCl containing 1mM EDTA-Na2. Firstly, the primer stock tube prepared and then the working solution would prepare from primer stock tube. According to the instruction provided by primer manufacturer (Bioneer / Korea) the TE buffer were added to get 100 Pico mole/ microliter concentration of primer stock solution. The working solution prepared from stock by dilution with TE buffer to get 10 Pico mole/ microliter and kept in -20 ºC.

2.2.6.4 The mixture of PCR reaction

Amplification of DNA was carried out in final volume of 25 µl containing the following as mentioned in Table (2-6)

Table (2-6) Contents of the Reaction Mixture

No.	*Contents of reaction mixture*	*Volume*
1.	master mix	12.5 µl
2.	Upstream primer	1.5 µl
3.	Downstream primer	1.5 µl
4.	DNA template	3 µl
	Nuclease free water	6.5 µl
Total volume		25 µl

2.2.6.5 Detection of *trichophyton species* by using specific primer

The oligonucleotide primers for genes used in this study were obtained from previous studies and summarized in table (2-7)

Table (2- 7) Primers sequences of *trichophyton spp* for *Trichophyton* detection and PCR condition

Trichohyton Spp	**Primer sequence (5'-3')**	**Size bp**	**PCR condition**	**Reference**
Trichohyton . rubrum	**F-RCGAGGAGAGGACCCRACHTC TGAC** **R- TTCCTTAGTACCRGCYTTG**	**925 bp**	**96 ºC 2 min 1x** **96 ºC 30S x30** **63 ºC 3 S** **74 ºC 120 S** **72 ºC 5 min 1x**	**ToshioKanbe ,. 2003**
Trichophyton . violaceum	**F-GATCCACAAGGTATGTTAGTTA** **R-GGTGCCAGCCATGTCGTAGAC**	**421 +925 bp**		
Trichophyton. mentagrophytes	**F GCCTGTTGTTCCGCTCATTCTT** **R CGGCTAGGAGGGCGTGGAGAA**	**392 bp**		

2.2.6.6 Detection of some virulence gene by PCR

DNA (extract from fungal cells) was used as a template in specific PCRs for the detection of some of *Trichophyton spp* virulence genes.The primer sequences and condition for PCR were summarized in table (2-8)

Table (2-8) Primer used in PCR assays for virulence gene of *Trichophyton spp*

Gene name	Primer Sequence	Product	PCR Condition	Study
Keratinase	F-CACTGGTGTCGATGTCAACC R- AGTTAATACCGGCGACGATG	227 bp	**94°C 5min X 1** **94°C 1min X 35** **57 °C 1min** **72°C 1min** ***72°C 1min 1x***	New design
phospholipase	F-GGAGACATCGATCAGGTGGT R- GATGATGCGGTCAAGGAACT	162 bp		
PacC	F-ACCAATTCCTGGAGCAGATG R- GTGGTGTGACTGGTGGTGAG	152 bp		
hsp90	F- TGAGTACGCCATGACTCAGC R- AACCTTCTCAACGGCATCAC	178 bp		

2.2.6.7 Multi Locus Sequence Typing (MLST)

In this study, The *Trichophyton species* MLST scheme uses internal fragments of the following seven house-keeping genes: internal transcribed spacer (*ITS*), Beta-tubulin (*BT2*), and translation elongation factor 1-a (*TEF-1a*), *CaM*, *HSP70*, *ACT*, and *D1/D2* .

Primer and PCR conditions were available on MLST database (https://www.frontiersin.org/articles/10.3389/fmicb.2021.643509).Was show in table (2- 9)

Table (2-9) Primer used in PCR assays for MLST of *trichophyton spp*

Gene	PCR Primers	PCR Product		
TEF-1α	F 5'- CACATTAACTTGGTC GTTATCG-3' R 5'- CATCCTTGGAGAT ACCAGC-3'	768 bp	95ºC 5 min 1 x 94 ºC 30 sec 30 x 58ºC 40 sec 72º C 5 min 72ºC 5 min 1x	Mithendi *et al*., 2014

BT2	F 5'-AACATGCGTGAGA TTGTAAGT -3' R 5'-ACCCTCAGTGTAGT GACCCTTGGC -3'	790 bp	95ºC 5 min 1x 94 ºC 30 sec 30x 56ºC 40 sec 72º C 5 min 72ºC 5 min 1x	Rezaei-Matehkolaei *etal 2014*
CaM	F 5'-TGTCCGAGTACA AGGAAGC -3' R 5'-TTACAATCAAT TCTGCCGTC -3'	664 b p	95ºC 5 min 1x 94 ºC 30 sec 30x 60ºC 40 sec 72º C 5 min 72ºC 5 min 1x	Ahmadi *etal .,2016*
ACT	F 5'-TCTTCGAGACC TTCAACGCC -3' R 5′-AAGCCACCGATC CAGACG -3′	687 bp	95ºC 5 min 1 x 94 ºC 30 sec 30x 55ºC 40 sec 72º C 5 min 72ºC 5 min 1x	Probst, *2002*
HSP70	F 5'-GTGGCTTCCCA GGTGCTG -3 R 5'-AATGATTTCAGTAAC CGACCC -3	600 bp	95ºC 5 min 1 x 94 ºC 30 sec 30x 55ºC 40 sec 72º C 5 min 72ºC 5 min 1x	Probst, 2002
ITS	F 5'-TCCGTAGGTGAACC TGCGG-3 R 5'-TCCTCCGCTTATTG ATATGC-3'	627 bp	95ºC 5 min 1x 94 ºC 30 sec 30x 58ºC 40 sec 72º C 5 min 72ºC 5 min 1x	White, *etal .,1990*
D1/D2	F 5'-GCATATCAATAAGCGGA GGAAAAG -3' R 5'- GGTCCGTGTTTCA AGACGG -3'	617 bp	95ºC 5 min 1 x 94 ºC 30 sec 30x 60ºC 40 sec 72º C 5 min 72ºC 5 min 1x	Kurtzmanet al ., (1997

2.2.6.8 Whole Genome Sequencing (WGS)

After extraction of genomic DNA from a new fungal growth on Sabouraud Dextrose Agar, the extracted DNA subjected to quantification by NanoDrop instrument to estimate the DNA concentration according to manufacturing's instructions. In addition, the condition of the DNA was assessed by gel electrophoresis method to evaluate the presence or absence of DNA in the sample, where 1µl of DNA loaded to 1% agarose gel and run at 160V for 30min. Following this step, only two successful sample was submitted to Macrogen company (Korea)

for WGS (Paired-ends) using the Illumina NovaSeq 6000 platform. The resulted raw reads were processed by further bioinformatics tools.

2.2.6.8.1 Bioinformatic Analysis

In this analysis, all bioinformatics approaches that used to analyze the study sequences were relied on either using command-line tools and bioinformatics softwares on open-source operating system, Linux (Version: Ubuntu 18.04.3 LTS, Canonical Ltd., UK), or using web-based servers such as the Galaxy platform (https://galaxyproject.org) (Afgan *et al*., 2018) **.**

Regrading WGS analysis, the raw data undergo quality control by FastQC (Version, 0.11.5) (Andrews, 2010) to evaluate quality of reads and calculating the basic statistics (such as total number of bases, reads and GC content). After quality control, raw reads were subjected to preprocessing steps to reduce biases in analysis by trimming out bases of low quality, adapter sequences and the Poly-G tail. Fastp (version 0.19.4) and Trimmomatic (version 0.36) tools were used for preprocessing steps (Bolger, Lohse, and Usadel, 2014; Chen *et al*., 2018). The filtered raw data have undergone for further processing steps included :

1- Mapping reads that are related to *Trichophyton Isolates* 6TR and 7TR to the reference genome by Burrows-Wheeler Aligner BWA, (BWAMEM) (v0.7.17) (Li, 2013) and assembly of these mapped reads to draft genome by IDBA-HYBRID - v1.1.3 v0.4. (Peng *et al*., 2012).

2 -BUSCO v5.2.2 (Simão *et al*., 2015) was used to provide a quantitative measures for the assessment of genome assembly.

3 -Regarding Repeat Masking of assembly genome, RepeatMasker v4.1.1. was used in this study (*Tempel et al*., 2012).

4 - For Gene Prediction and annotation, GeneMarkES v4.48 (Borodovsky *et al*., 2011) was used for gene prediction and STRING v11 (Szklarczyk *et al*., 2019) was used for genome annotation.

5 - For identification of ORF, tRNA, rRNA and SSR, Getorf vEMBOSS 6.6.0, tRNAScan-SE v2.0.7, RNAmmer v2, SSR Finder were used respectively according to Humann *et al*, (2019).

6 -Multiple genome alignment was used by Mauve (v2.4.0) (Darling *et al*., 2010) .

7 -Variants calling was done by SAMTools (Li, 2011); Variant annotation type and impact was done by SnpEff (v4.3t) (Cingolani *et al*., 2012).

8 -Regarding phylogeny, maximum likelihood method was used to build the tree by MEGA11 software according to core genes extracted by STRING v11 (Szklarczyk *et al*., 2019).

2.2.6.9 Detection of Amplified Products by Agarose Gel Electrophoresis

Successful PCR amplification was confirmed by agarose gel electrophoresis (Lodish *et al.,* 2004). Agarose gel was prepared by dissolving 0.6gm of agarose powder in 5ml of TBE buffer (pH:8) then the volume completed to 50 ml deionized water, in boiling water bath, allowed to cool to 50^{o}C and green star at the concentration of 5µl/ml was added.

The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was powered gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray.

The tray was fixed in an electrophoresis chamber which was filled with TBE buffer covering the surface of the gel, 5µl of DNA sample was transferred into the signed wells in agarose gel, and in one well we put the 5µl DNA ladder mixed with 1µl of loading buffer. The electric current was allowed at 70 volt for 30 min.

3.1. Isolation and Identification of *Trichophyton species*

3.1.1 Isolation of *Trichophyton species*

A total of 150 specimen (patient) were obtained from individuals who were diagnostic clinical dermatologist to have a cutaneous fungal infection, including scrapings of the skin (n = 100) and nails (n = 50). The Patients were visited to dermatology outpatient clinic of Marjan teaching hospital , and dermatology outpatient clinic Al‑Sadeq teaching hospital , these specimens diagnostic was done clinically and Full information were detected from each patient including : age ,sex ,clinical type and severity by dermatologist between April and October 2022. as shown in Table (3-1). All sample were subjected to aerobic culturing on sabouraud dextrose agar and it was out of the total 150 specimen , 90 specimen showed positive fungal culture divided to (70) skin specimen and (20) nail specimen . No growth was seen in other (60) specimen which may be due to the presence other skin or nail disease , misdiagnosis or other causes. Among (50) positive culture of *Trichophyton species* , only 27 specimen show positive was the method by which culture were considered positive identified as *Trichophyton* . *mentagrophytes ,Trichophyton rubrum and Trichophyton violaceum*.

These isolates subjected to molecular detection method using specific primer for confirmed isolation of *Trichophyton species* by PCR . The results revealed that only 27 out of 50 were positive for PCR as shown in table (3-1) .

Table (3-1): Prevalence of *Trichophyton species* among other etiological agents associated with specimen isolated

No of sample	**Negative culture**	**Positive culture of *dermatophytone***	**Positive ctulture of *Trichophytone species***	**No. moleculer positive**	No moleculer negative
150	60	90	50	27	23

Bhatia and Sharma (2014) were found *Trichophyton species* were implicated in 98.6% . Among the dermatophyton , *T. mentegrophyte* was the predominant organism followed by *T. rubrum* .Also , Fernanda Cristina *et al*,.(2019)were found most common infecting was *Trichophyton species* .

Determining the prevalence of *Trichophyton species* among other etiological agents associated would require access to specific epidemiological data or studies conducted in a particular region or population. Prevalence rates can vary depending on geographical location, population demographics, and sampling methods used.

The parameters affecting the high incidence of dermatophytoses that need to be indicated are independent on both the host and the pathogen, these are mainly climatic conditions such as humidity and temperature. *Trichophyton* grow best in warm and humid environments; hence, they are more common in tropical and subtropical regions. Therefore, dermatophytoses are more prevalent in Iraq , Libya, Iran, Turkey, Ethiopia, Uganda, Italy and other warm countries than in countries with colder climates (Kakande *et al* ., 2019) .

In Asia , whereas *T. rubrum, T. mentagrophytes, and Trichophyton violaceum* are the most frequently isolated pathogens in these regions (Seebacher *et al* ., 2008).

3.1.2 The identification *Trichophyton species*

3.1.2.1 The Characterization of *Trichophyton species*

The identification of *Trichophyton species* depends on the microscopic patterns and cultural also biochemical characteristics. But these characteristics cannot be utilized to distinguish one species from another due to the variability that the fungus can display in its morphologic characteristics with subcultures; therefore, more accurate methods, such as molecular tests, must be used. However, some characteristic of *Trichophyton species* should be considered to confirm the identification of this fungal through using specific markers via polymerase chain reaction techniques.

3.1.2.2 Direct microscope examination

Direct microscopic examination of skin and nails is the most rapid method of determining fungal etiology and is traditionally accomplished by examining the clinical material in 10% potassium hydroxide (KOH) (Kane *et al* .,1997).

If KOH Test -positive samples are taken into account positive. Several samples that were positive for *Trichophyton species* in KOH were found to be negative for culture .These negative results are likely due to the fact that patient taken antifungal, used cream in site of infection, The sample may have been contaminated during collection or processing, leading to inaccurate results, The sample may have been taken from an area where the fungal infection was not actively growing (Saunte *et al* ., 2020).

3.1.2.3 Culturing of *Trichophyton species*

Differential diagnosis of fungal colonies of *Trichophyton rubrum, Trichophyton mentagrophytes, and Trichophyton violaceum* can be based on various factors, including the macroscopic and microscopic appearance of the colonies, growth characteristics, and biochemical tests. Macroscopic appearance on sabouraud agar : *Trichophyton mentagrophytes* colonies are usually white or

yellow and appearance of colonies (powdery or velvety) ,while *Trichophyton rubrum* colonies typically appear white, pink, or red and culture reverse pigmentation may vary from colourless to yellowish to yellow-brown to wine red , colonies that characteristic culture surface texture may vary from downy to suede-like; and *Trichophyton violaceum* colonies are usually dark purple or violet .

Microscopic appearance: Examination of the fungal hyphae and spores under a microscope can also help distinguish between these species. *Trichophyton mentagrophytes* hyphae are often thick and twisted, with spores that are usually solitary. *Trichophyton rubrum* typically has short, curved hyphae and spores that are often grouped in clusters. *Trichophyton violaceum* hyphae are often long and straight, with spores that are typically in chains.

Growth characteristics: *Trichophyton mentagrophytes* colonies grow more rapidly, producing visible growth within 5-7 days . *Trichophyton rubrum* colonies typically grow slowly, taking about 10-14 days to produce visible growth. *Trichophyton violaceum* colonies grow more slowly than either *T. rubrum or T. mentagrophytes,* taking up to 21 days to produce visible growth.

Also , Numerous microconidia are borne laterally along the sides of hyphae, and are predominantly slender clavate when young. With age the microconidia become broader and pyriform to spherical in shape. Occasional to moderate numbers of smooth, thin-walled, multiseptate, clavate to cigar-shaped macroconidia may be present. Varying numbers of spherical chlamydospores and spiral hyphae may also be present.

Growth on SDA with chloramphenicol, gentamycin and cycloheximid were incubated at (25 °C ± 5 °C) for up 3- 4 weeks and observed regularly for growth. Colonies of *Trichophyton species* on SDA may be velvety, powdery or waxy with pigmentation shown by some species and may be readily pushed intact over the surface of agar using a sterile loop (Alex and Sue , 2022).

Biochemical tests: Various biochemical tests can also be used to distinguish between these species. For example, *Trichophyton rubrum* and *Trichophyton mentagrophytes* can be differentiated based on their ability to metabolize urea and hair perforation test. *Trichophyton violaceum* can be identified based on its ability to produce the enzyme tyrosinase

The diagnostic features of *Trichophyton* are summarized in Table(3-2).

Table (3-2): The most important traditional test used.

Trichophyton species	***Trichophyton .mentagrophytes***	***Trichophyton .rubrum***	***Trichophyton . violaceum***
Hyphae	are often thick and twisted, with spores that are usually solitary	are short, curved hyphae and spores that are often grouped in clusters.	are often long and straight, with spores that are typically in chains
Growth	colonies grow more rapidly, producing visible growth within 5-7 days.	colonies typically grow slowly, taking about 10-14 days to produce visible growth.	grow more slowly, taking up to 21 days to produce visible growth
Colony on Sabouraud dextrose agar	colonies are usually white or yellow	colonies typically appear white, pink, or red yellow-brown to wine red.	colonies are usually dark purple or violet
Colony on Potato Dextrose Agar	colonies are initially white or cream-colored, becoming yellowish or tan with age.	colonies are initially white or cream-colored, which later turn to pale yellow or pinkish-tan.	colonies are generally whiteor cream-colored, often developing a lavender or violet pigmentation.
Dermatophyte Test Medium	colonies are typically cottony or powderyin texture. Initially, the colonies are white or cream-colored, but they may turn	The colonies are usually cottony or fluffy , develop a reddish-brown or pinkish color at the center of the colony.	colonies are white or cream-colored, but they may turn yellow or tan a Reverse side: The reverse side of the colony may

	yellow or tan as they mature. The reverse side of the colony may exhibit a yellow or reddish-brown color.	reverse side of the colony may turn red or pink	exhibit a yellow or reddish-brown color.
Corn meal agar	colonies can be white, cream, or yellowish in color. The reverse side of the colony may exhibit a yellow or reddish-brown color	Colonies can be white, cream, or pinkish in color. Reverse side: The reverse side of the colony may exhibit a red or brown color..	The colonies can be white, cream, or have a lavender or violet pigmentation.The reverse side of the colony may exhibit a similar violet or lavender color.
Bromocresol purple (BCP)	colonies are cottony or woolly texture. The color can vary from white to yellow or reddish-brown. The colonies are typically circular or irregular in shape, with undulating or lobed edges	colonies are cottony or powdery texture. They can have a range of colors, including white, cream, yellow, or pink. The colonies often appear circular or rounded with smooth edges	produces weak growth accompanied by clearing of the milk solids and a purple colour change
Urease test	Positive	Negative	Positive or Negative
hair perforation test	Positive	Negative	Negative
Nitrate reduction	Negative	Negative	Positive
The Tween 80 Hydrolysis	Positive	Negative	Negative
Acid production test	Positive	Positive or negative	Positive
Indole production test	Positive	Negative	Positive

3.1.2.4 Confirmed diagnosis of *Trichophyton species* by PCR using multiplex PCR

The one purpose of this study was to develop molecular diagnostic test to identify and purity of *Trichophyton species* isolates based on the specific primer profiling, comparison with of traditional criteria and that may be good condition to be used as genotyping (marker) for confirmatory identification of *Trichophyton species* from patients.

The DNA was extracted from 50 specimens was collected from skin and nail scraping which used in conventional PCR.It was carried out using the DNA samples for amplification of specific primers ; according to the sequences and program listed in Table (2-7). After that gel electrophoresis showed that , only 27 produced the specific base pair DNA fragment when compared with allelic ladder, as shown in figure (3-1) table (3-3) .

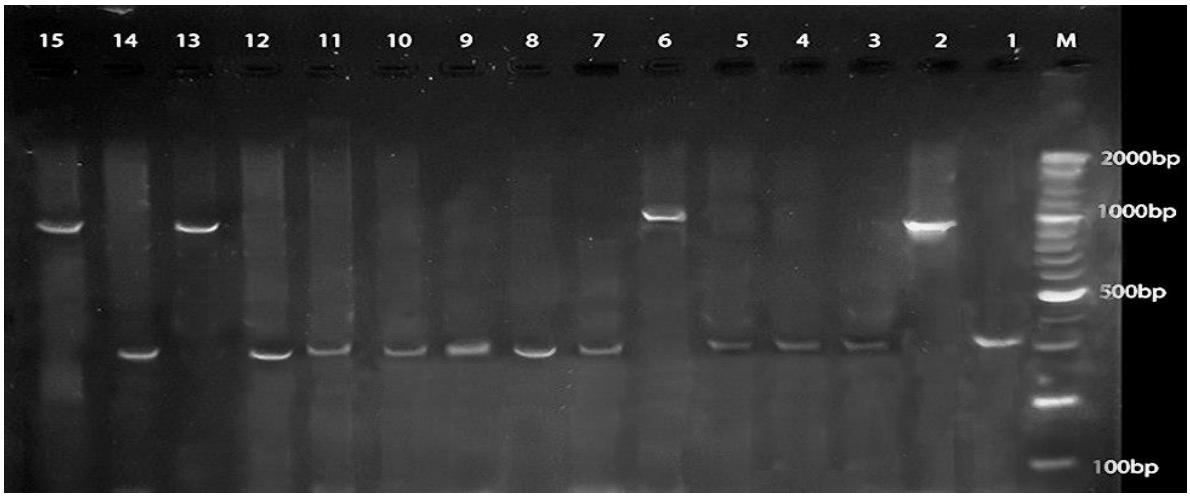


Figure3- 1: The electrophoresis of agarose gel of multiplex-PCR products obtained with *Trichophytones species* -specific primers that generated: 925bp =*T. rubrum*, 392bp= *T. mentagrophytes*. Lane M is a 100bp DNA ladder. Lanes 1,15 from nail and skin. The electric current was allowed at 70 volt for 30 min , 1.5 % agarose

Table 3-3 Detection of *Trichophyton species* by using specific primers

Number of sample detected by PCR	Number of Trichophyton spp detected by PCR	*Trichophyton spp*	Number (%)
50	27	*T. rubrum*	**7 (25.93%)**
		T. violaceum	**4 (14.81%)**
		T. mentagrophytes	**16 (59.26%)**
			27 (100%)

In this study, demonstrated PCR targeting of the fungal DNA for identification of the common *Trichophyton species* including *T. rubrum, T. mentagrophytes* and *T. violaceum* but not from any other fungal species. According of the result above the *Trichophyton spp* depend on multiplex PCR it was observed that only (27) isolates of *Trichophyton spp* out of (50) were decomented as *Trichophyton species* by culture and biochemical test . However ,the result of this study was found most commen infection by *Trichophyton mentagrophytes* 59.26% following by *Trichophyton rubrum* 25.93% and *Trichophyton violaceum* 14.81 % .

The specific primer used to investigate the presence *Trichopytones species* . The clinical samples of patients suspected of having a cutaneous fungal infection contained a total of 50 DNAs were extracted.These DNAs were detected by the multiplex PCR designed in this work to simultaneously detect the infection and identify the causative organisms .

Dhurgham *et al* ,. (2022) who were found the skin lesions with the common dermatophytes specieswas identified as *Trichophyton mentagrophytes* (75%) Also, Qadisiyah and Alaa., (2023) were found *Trichophyton mentagrophytes*

isolates were the most common fungus isolated (67.5%), indicating a significant prevalence of this fungus causing dermatophytosis .

On the other hand ,the result obtained by Amin *et al* ., (2022) who were found the percentage *Trichophyton mentagrophytes* (59 %) was the most common isolate followed by *Trichophyton rubrum* (10.9%).

Also Li J *et al* .,(2018) ; Jain *et al* .,(2020) who found the *T. mentagrophytes* surprisingly turned out to be the most common dermatophyte with prevalence of up to 75.9 to 77.5% followed by *T. rubrum*. Namrata *et al* ., (2022) who found that infection with *T. mentagrophytes* about 84.6 % following *Trichophyton rubrum* (7.6%).

On other study in Greece found *Trichophyton Species* by molecularly identified clinical isolates (70%) *T. rubrum*, (24 %) *T. mentagrophytes*, (12 %)*T. interdigitale* and (6%) *T.tonsurans* (Maria *et al* ., 2021).

Also , other study in Iran the species of Trichophyton were identified as *Trichophyton mentagrophytes* (24.5%), *Trichophyton rubrum* (5.9%), *Trichophyton violaceum* (4.2%) (Keyvan and Jamal 2006).

The higher frequency with *Trichophyton mentagrophytes* may be due to the direct or indirect contact with domestic animals such as cattle and cat because they are zoophilic fungi and cause many ring worm infections.

Tinea corporis is a common type of dermatophytosis that infects smooth skin, except for the scalp, hair, palms, nails, and genital area. When the pathogens invade the stratum corneum of the skin, they cause a mild inflammatory reaction, consisting of erythema, papules, and blisters, followed by ringworm lesions with obvious scales. The source of the infection is typically contact with contaminated items, infected animals.

The geographical origin of the strains has remained difficult to ascertain. Increased travel and migration of people has been regarded as a contributing factor.

Various tests based on histology, colony characteristics, ability to hydrolyze urea, hair perforation, PCR, and sequencing have been improved upon to detect different dermatophytes. While molecular techniques are often more reliable than conventional methods of identification, PCR methods have been shown to vary between investigators (Verrier and Monod , 2017) .

On the other hand , *T. violaceum* is simply a phenotypically different strain from *T. rubrum* that has arisen due to differences in physiological stress occurring in different habitats, because it is crucial to consider that virulence and adaptation are also essential for definition of the species when determining the correct affiliation of the species and the limits between them (Angélica *et al* ., 2023).

Culture failed to find any mixed Trichophyton infections in the study's sample material. Sampling variance is a more likely explanation for this result because both *Trichophyton* needed multiple samples in order to thrive in the culture. Another argument is that in cultured samples of various *Trichophyton species*, the more common *Trichophyton* species will probably perform better than the less frequent ones .

If culture-positive samples are taken into account as true positive , a multiplex PCR test is just as effective as conventional diagnostic techniques. Several samples that were positive for *Trichophyton species* in culture were found to be negative for multiplex PCR. These negative results are likely due to the fact that trichophytosis-causing agents in these samples came from species that weren't considered in this multiplex PCR. It is important to highlight that such sample division issues have long been recognized as a potential problem in dermal mycology analysis (Aylin *et al* ., 2008) .

However, because this methods of classification and identification takes time, it could be difficult for non-experts to tell apart the morphology of cultured colonies. Additionally, even the identical strains can result in morphologically

different colonies, making it more difficult to identify the causative agent. (Ji Young Kim *et al* ., 2011) . So that confirm diagnosis of *Trichophyton species* by used specific primer .

However, some characteristic of *Trichophyton* should be considered to confirm the identification of this fungal through using specific markers via PCR techniques

So that , Fungal cells can be identified using as little as 1 pg of DNA because to the PCR method's high sensitivity and specificity. Multiplex PCR, which uses more than two primers for simultaneous amplification and is widely used in clinical microbiology, allows for the detection of several species from a single specimen. But when using the PCR method, it's crucial to select the primer and focus on the DNA that has to be amplified. When creating the primer, it is important to take into account the recurring base sequences that are shared by all dermatophytes, random base sequences with a high possibility of binding, and various specific base sequences found on a single chromosome (Ebrahimi *et al* ., 2019) .

The results obtained from the PCR coincided with this of the traditional morphological identification techniques .This indicates that PCR targeting gene is able to be applied as a rapid identification of clinical specimens from patients . These study concerning the strain identification provide useful information for the surveillance of the disseminations of dermatophyte fungi in environments , including hospital .For the study of strain identification of dermatophytes ,a large number of fungal isolates should be identified correctly and rapidly to a species level (Kanbe, 2008) .

The varying frequency of dermatophytes identified can be explained by the evolution of urban and rural populations, the growing number of companion animals, and humidity and temperature, among other factors. These factors

influence dermatophytes' phenotypic and genotypic diversity in different geographical areas (Bontems *et al* ., 2020).

3.2 Distribution of *Trichophyton species* according to site of infection

In this study , A total of 150 specimen (patient) were obtained from individuals who were diagnostic clinical dermatologist to have a cutaneous fungal infection, including scrapings of the skin (n = 100) and nails (n =50)as show in table (3 – 4) . The Patients were visited to dermatology outpatient clinic of Marjan teaching hospital , and dermatology outpatient clinic Al‑Sadeq teaching hospital , these specimens diagnostic was done clinically and Full information were detected from each patient by dermatologist, between April and October 2022.

Table 3- 4 Distribution of *Trichophyton species* according to site of infection

Sources of isolates	No. of samples	No. of *Trichophyton species* isolates	
		count	Percentage
Skin	100	20	74.1%
Nail	50	7	25.9%
Total number	150	27	100%

This table indicates that the rate of *Trichophyton species* most common isolated from skin than nail was 20 (74.1 %) .

Lim Chui Ng *et al* ,. (2023) who were found the prevalence of *trichohyton* is approximately (58 %) isolate from skin and (33.3 %) isolate from nail .

On the other hand (Hiba and Mouna ., 2022.) who were found (46 %) isolate *Trichophyton species* from skin and (23%) isolate from nail .There are several reasons for skin infection more that nail infection ,first The skin is constantly exposed to the environment and can be easily damaged by cuts, scrapes, and other injuries, which can provide an entry point for fungal spores. In contrast,

the nails are protected by the surrounding skin and are less likely to be damaged or exposed to fungal spores. Second The skin is warm and moist, which provides an ideal environment for fungal growth. In contrast, the nails are cooler and drier, which makes it more difficult for fungi to grow and multiply. However, it is important to note that *Trichophyton* nail infections can also occur and can be more difficult to treat than skin infections (Gupta and Versteeg *et al* ., 2021).

The isolation of *Trichophyton species* from skin and nail depends on several factors like antifungal , personal hygiene ,effect of detergents , health status of patients and also the effect of environmental conditions temperature and humidly. The incidence of fungal skin diseases has increased , and this may be due to several reasons, for example, the increase in the number of people who are sensitive to these diseases, such as the elderly, the immunocompromised, the low social status, and various activities such as sports, as well as wearing low-ventilated shoes, using shared swimming pools, and increasing immunodeficiency patients, as well as diabetes, antibiotics and taking immunosuppressive drugs (Martinez DA *et al* ,. 2012).

Li, C *et al* ,. (2022) who were found most common infection in skin .Also (Gaurav *et al* ., 2022) found The commonest clinical type was Tinea corporis (48%) . Algimantas and Švedienė ., (2013) who were found the prevalence of *Trichophyton* infection in nail (75.5 %) more than in skin(11.3 %). Variations in site of infection between studies can be attributed to a variety of factors, including sanitary practices in hospitals and staff ,their geographical regions, environmental conditions, isolation and identification techniques, social and cultural level of patients, and use of multidrug (antifungal) that may lead to fungal resistance development , all of these factors may combine and play an important role in inhibiting or stimulating fungal resistance development Cutaneous fungal infection of the skin and nails poses a significant global public health challenge.

Dermatophyte infection, mainly caused by *Trichophyton species* , is the primary pathogenically agent responsible for skin and nail infections worldwide. The epidemiology of these infections varies depending on the geographic location and specific population (Pattriya *et al* ., 2023) .

The primary causative agents of tinea corporis worldwide in recent years are *Trichophyton spp* with *T. rubrum* and *T.mentagrophytes* (Bunyaratavej *et al*., 2019).

Trichophyton are pathogenic fungi with high affinity for keratinized structures present in nails and skin causing superficial infections known as dermatophytoses. *Trichophyton species* are associated with the type of species-specific host keratin with the highest sensitivity, which is the main reservoir of the pathogen .The remarkable ability of this group of fungi to survive in different ecosystems results from their morphological and ecological diversity as well as high adaptability to changing environmental conditions (Gnat *et al* ., 2019) .

Moreover, one lesion can be caused by a single strain or species of *Trichophyton* , but it is also possible to identify many various types, strains and species of fungi in the same lesion . Additionally, a single species of *Trichophyton* can cause different types of clinical lesions like tinea corporis and tinea unguium (Arastehfar *et al* ., 2019)

3.3 Distribution of *Trichophyton species* according to age Categories

Table (3 - 5) Distribution of *Trichophyton species* according to age Categories

Age	No of sample	No. of *Trichophyton species* isolates	
		Count	Percentage
17 – 33 year	30	5	18.52 %
34 – 44 year	35	7	25.93 %
45 – 65 year	85	15	55.55%
Total	150	27	100 %

Table (3-5) indicates that the rate of *Trichophyton species* most common group involved was 45 – 65 year 15 (55.55 %) .

Gaurav Saxenaa *et la* .,(2022) who were found The age of 21 - 40 years was most commonly affected (55 .4 %) Also, (Majid Rauf *et la* ., 2021) who were found the age group of 31-40 (25%) most common infection . The findings of this study were consistent with those of numerous previous studies; however, because sample sizes varied, there were a few modest and significant discrepancies at certain times.

On the other hand Several factors contribute to the higher prevalence of *Trichophyton* infections in the elderly population, first : weakened immune system, As individuals age, their immune system undergoes natural changes, leading to a gradual decline in immune function. This age-related decline, known as immunosenescence, can result in a decreased ability to mount an effective immune response against fungal infections. The weakened immune system makes older individuals more susceptible to *Trichophyton* infections (Hof , 2010) .

Second : chronic health conditions: older adults often have higher rates of chronic health conditions such as diabetes, peripheral vascular disease, and peripheral neuropathy. These conditions can compromise the blood supply to the skin, impair nerve function, and contribute to reduced sensation in the extremities. Such compromised circulation and decreased sensation increase the risk of fungal infections, including those caused by Trichophyton.

3.4 Distribution of *Trichophyton species* according to sex Categories

Table (3-6) Distribution of *Trichophyton species* according to sex Categories

sex	No of sample	No. of *Trichophyton species* isolates	
		count	Percentage
Male	105	17	62.96 %
Female	45	10	37.04%
Total	150	27	100%

Table(3-6) indicates that the rate of *Trichophyton species* most common isolated from male 17 (62.96 %) than female 10 (37.04 %)

(Khalifa *et al* ., 2021) who were found the most common gender group was male (59.6%) male and (40.4%) females with varying types of dermatophyte infections.

This difference between *Trichophyton species* infection and gender of the patients may be due to physiological differences between male and female , For example, males tend to have more body hair, including hair on the chest and back which provides a favorable environment for fungal growth and colonizatio .Male-specific activities and occupations may also contribute to a higher risk of exposure. Jobs involving prolonged exposure to moisture, such as construction work or

agriculture, can increase the likelihood of fungal infections and the differences in the social behavior (Vella *et la* ., 2003).

On the other hand , hormonal differences between males and females have been suggested as a contributing factor. Androgen hormones, more abundant in males, can influence sebum production, sweat gland activity, and skin pH, potentially creating a more favorable environment for fungal growth. Karlre *et al* ., (2010) Bhavsar *et al* ., (2012) Also, Males are more numerous than females because most girls prefer to visit outpatient clinics (Hiba and Mouna ., 2022) .

3.5 Distribution of *Trichophyton species* according to clinical manifestations

Table (3 - 7) Distribution of *Trichophyton species* according to clinical manifestations

Clinical manifestation	No of sample	No. of *Trichophyton species* isolates	
		Count	**Percentage**
Tinea corporis	90	15	55.55
Tinea cruris	40	5	18.52
Tinea unguium	20	7	25.93
Total	150	27	100

Table(3-7) indicates that the rate of Tinea corporis most common was a higher prevalence at about 15 (55.55%) followed Tinea unguium about 7 (25.93 %) and following Tinea cruris about 5 (18.52%).

Khalifa *et al* ., (2021) who were found tinea corporis was the main fungal infection observed (35%) of cases , tinea cruris (5.6%) and tinea unguium (0.8%) . Also Gaurav *et al* ,. (2022) who were found the most common clinical

type was tinea corporis(48%) followed by tinea unguium (18%) and tinea cruris (14%) .And Seeba Hussain ,(2023) who was found Tinea corporis was observed to be the most prevalent clinical form (61.20%), followed by tinea cruris (24.34%).

The increased prevalence of tinea corporis can be attributed to overcrowding, sharing of personal hygiene equipment including towels. In addition, some social behaviors in the Iraqi community, handshake can play a role in spreading infection as contact is an important portal for transmission of infection (Brook *et al* ., 2001) .

Tinea corporis, tinea cruris, and tinea unguium are among the most common fungal infections because they share certain characteristics that contribute to their prevalence,first : fungal Environment , The fungi that cause these infections thrive in warm, moist environments. Areas such as the skin, groin, and nails can provide suitable conditions for fungal growth, especially when there is increased moisture, sweating, or poor ventilation. These favorable conditions make the skin and nails susceptible to fungal colonization and infection (Debra *et al* ., 2023) .Second : direct contact transmission ,Tinea infections are typically spread through direct contact with infected individuals or contaminated surfaces. The fungi can be transmitted through skin-to-skin contact or by sharing personal items such as towels, clothing, or sports equipment. This mode of transmission facilitates the spread of the infection, particularly in close-contact settings such as households, schools, gyms, and communal areas. Third : compromised skin or nail barrier: The fungi that cause tinea infections can penetrate and infect the skin or nails when there is a disruption in the protective barrier. Factors such as minor skin injuries, excessive moisture, weakened immune system, or underlying conditions like diabetes can compromise the skin or nail integrity, making individuals more susceptible to fungal invasion .And fourth : shared Risk Factors: Tinea infections share common risk factors that contribute to their prevalence. These include poor

personal hygiene, excessive sweating, wearing tight or non-breathable clothing, living in crowded or communal settings, participating in sports activities, having a compromised immune system, or having a history of previous fungal infections (Jain *et al* ., 2010).

3.6 Molecular Detection of some virulence genes in *Trichophyton species*

3.6.1 Molecular Detection of *keratinase gene* in *Trichophyton species*

It was found that 26 (96.29%) isolates gave positive result with this primer as shown in Figure (3-2) and table (3- 8) . Almost *Trichoytones species* isolated have keratinase gene .Proteolytic enzymes necessary for the breakdown of keratinic waste products are produced by *Trichophyton spp*

Table (3 – 8) detection of keratinase gene in *Trichophytones species*

TTrichophytone species	**Number N=27**
T. mentagrophytes	15/16
T. violaceum	4/4
T.rubrum	7/7
Total	26 (96.29%)

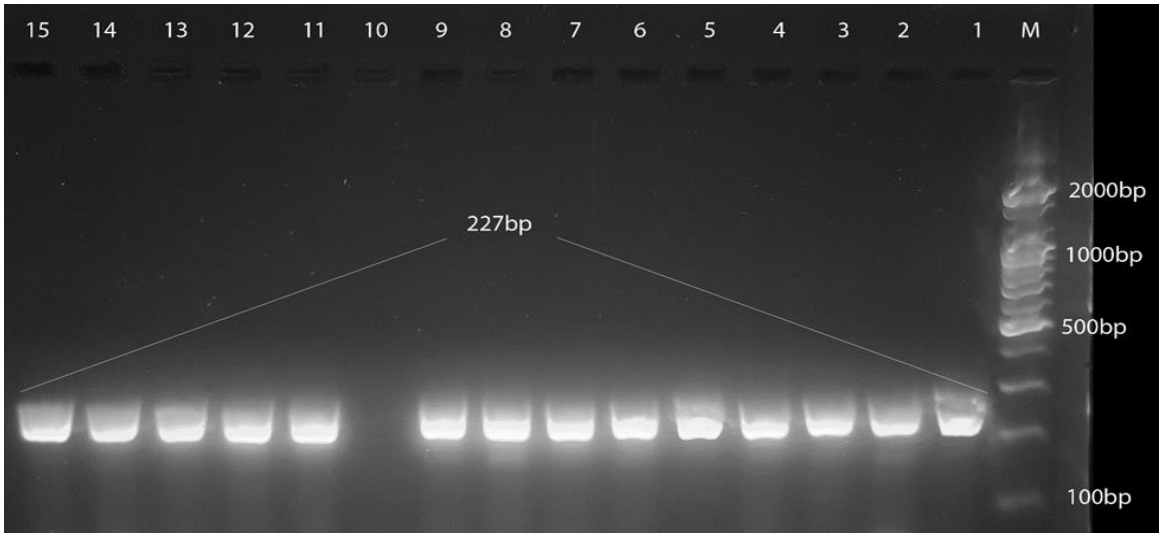


Fig. (3- 2): The electrophoresis of agarose gel of PCR products acquired with keratinase-specific primers that generated 227bp. lanes (1,2,3,4,5,6,9,11,12,13,14,15) were *Trichophyton mentagrophytes* ,lanes(8) were *Trichophyton rubrum* and lane (7) was *Trichophyton violaceum*.The electric current was allowed at 70 volt for 30 min , 1.5 % agarose

Abbas (2020) who were found *keratinase gene* was present only in (50%) isolates of *Trichophyton spp*. The present or absence of gene was related with taxonomic affiliation and origin of isolates. However, keratinases is expressed by common *Trichophyton , T. rubrum* and *T.mentagrophytes* which have optimum activity at acidic pH values, and are able to establish contact with human acidic skin due to the environment (Martinez *et al* ., 2012).

The secretion of keratinases and have been identified as an important step in fungal pathogenicity and virulence. The metabolism of some amino acids shifts the extracellular pH from acidic to alkaline values at which most known keratinolytic proteases have optimal enzymatic activity. This is a fundamental mechanism observed in *T. rubrum and T. mentagrophytes* in establishing superficial infection (Peres *et al* ., 2010).

The presence or absence of the keratinase gene, as well as the specific variations in its sequence, can help in understanding the diversity of *Trichophyton* species and their ability to cause infections. The prevalence of keratinolytic fungi

depends on different factors such as the organic matter, dissolved oxygen concentration, creatinine contents and environmental factors such as temperature, pH, and geographical location . PH is the most important factor, which markedly influence enzyme activity. Extremely high and low pH values generally complete loss of activity for most of enzymes (Khan and Bhadauria , 2015) .

Soft keratins, typical of the stratum corneum, contain relatively disordered protein filaments with fewer disulfide linkages (∼2–4% cysteine) ,as well as other linkages, for example, isopeptide (gamma-glutamyl–epsilon-lysine) bonds. Over 144 different proteins have been detected in human nails including keratins, cytoplasmic and junctional proteins. In the skin, proteins associated with keratin include elastin, collagen, fibronectin, laminin and filaggrin . The summarize the current state of the art of keratin biodegradation by dermatophytes and its links with virulence, focusing specifically on sulfitolysis and proteolysis.(Monod *et al* ., 2014) .

The keratinophilic fungi are of prime importance in regard to various skin diseases prevalent in various areas. These fungi are able to utilize keratin, a fibrous protein, as sole carbon and nitrogen source and survive saprophytic ally in nature . Many keratinophilic fungi frequently parasitize keratinous tissue skin and nails in man (Salil singh ., 2019) .

The Fungal extracellular enzymes assist to harm keratin's macromolecules into micro molecules which they can take in. For it, they require carbon substance as a deliver of electricity and nitrogen substance to gather protein and each exquisite critical compound . Dermatophytes rely upon keratin as a source of nutrition. Ancestral dermatophytes gleaned this keratin primarily from soil, but recent adaptive radiation of this lineage has resulted in fungi that are adapted to keratins of specific host species (Zheng *et al* ., 2020).

3.6.2Molecular Detection of phospholipase gene in *Trichophyton species*

Phospholipase gene was investigated by PCR technique using specific primers for this gene. It was found that phospholipase gene marker was observed in 27(100%) isolated which included 20 isolates from skin and 7 isolates from nail . As shown in figure (3- 3) and table (3- 9).

Table (3- 9) detection of phospholipase gene in *Trichophyton species*

Trichophytone types	Number N=27
T. mentagrophytes	16/16
T . violaceum	4/4
T.rubrum	7/7
Total	27 (100%)

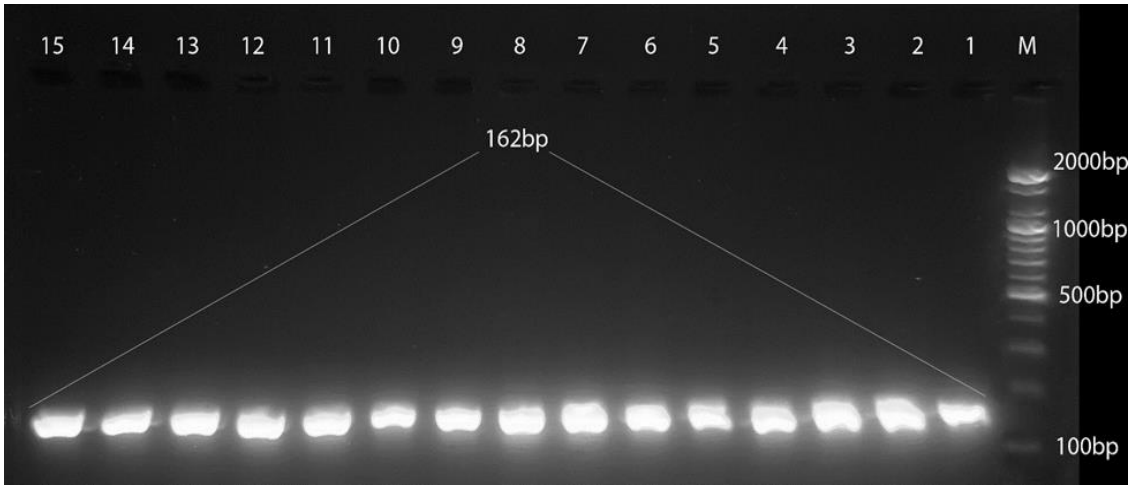


Figure 3- 3 : Agarose gel electrophoresis of PCR product acquired with phospholipase-specific primers that generated 162bp. Lane M is a 100bp DNA ladder . lanes (1,2,3,4,5,6,9,11,12,13,14,15) were *Trichophyton mentagrophytes* ,lanes(8,10) were *Trichophyton rubrum* and lane (7) was *Trichophyton violaceum* . The electric current was allowed at 70 volt for 30 min , 1.5 % agarose.

Elangovan *et al* ., (2017) Who were found all *Trichophyton* isolated (100 %) has phospholipase. Also (Faezeh Mohammadi *et al* ., 2022) who were found all *Trichophyton* isolated (100 %) showed the ability to produce phospholipase. This enzyme in the imoportant stage of dermatophytosis infection and fungal colonization. Phospholipases, as one of the virulence markers, facilitate the colonization of host cells by attaching the fungus to the target tissue and destroying the cell membrane following the hydrolysis of phospholipids

However , positive result of this gene will render the fungal to ability of *Trichophyton species* to produce phospholipase gene to cause cell lysis by cleaving phospholipids, which destabilizes the membrane. The membrane disruption activities that take place during host cell invasion are believed to involve phospholipase which is an important step in infection .

The function of phospholipase is demonstrated by their roles in colonization of host by attaching the *Trichopyton* to the target tissue and destroying the cell membrane following the hydrolysis of phospholipids. These enzymes may play an important role in balancing host immunity and the ability of *Trichophyton* to reduce the immune response(Ghannoum and Elewski 2004) .

3.6.3 Molecular Detection of Transcription factor PacC gene in *Trichophyton species*

Molecular detection of *PacC* gene was done by using specific marker. .It was found that 27(100 %) isolates gave positive result with this primer which included 20 isolates from skin scrape and 7 isolate from nail scrape as shown in Figure (3-4) and table (3-10)

Table (3 -10) Detection of *PacC gene* in *Trichohyton species*

Trichophytone species	Number N=27*
T. mentagrophytes	16/16
T. violaceum	4/4
T. rubrum	7/7
Total	27 (100%)

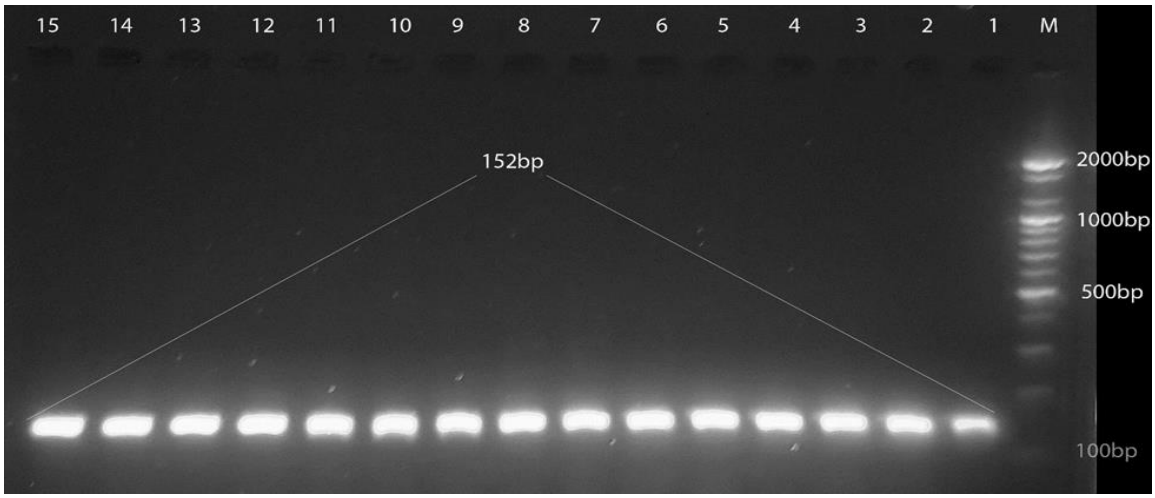


Fig.(3-4): Agarose gel electrophoresis of PCR product acquired with transcription factor PacC-specific primers that generated 152bp.. Lane M is a 100bp DNA ladder. lanes (1,2,3,4,5,6,9,11,12,13,14,15) were *Trichophyton mentagrophytes* ,lanes(8,10) were *Trichophyton rubrum* and lane (7) was *Trichophyton violaceum* . The electric current was allowed at 70 volt for 30 min , 1.5 % agarose.

Trichophyton regulate their gene expression to overcome host resistance and acidic skin pH, and this is crucial for cell rescue, defense, and virulence. The identification of genes upregulated in *Trichophyton* at acidic or alkaline pH may reveal the infection strategy developed by the pathogen. The unigenes upregulated at acidic or alkaline pHs encode proteins putatively involved in diverse cellular processes, such as metabolism, cell rescue, defense, virulence, cellular

communication, signal transduction,and fungal pathogenicity(Henrique *et al* ., 2010) .

Inactivation of the *pacC gene*, a component of the pH signalling pathway in *T. rubrum*, reduces the activity of secreted keratinases , indicating that the *pacC* gene is somehow involved in the regulation of keratinolytic activity, and consequently in the virulence and pathogenicity of this organism (Ferreira *et al* ., 2006) .

The transcriptional response of *Trichophyton* subjected to environmental conditions including pH variations in order to identify additional parameters that contribute to infection . One of the reasons that led to the spread of this species to the rest of the species because it generates a small immune response with large numbers of mycelia and the possession of the pathogen for some enzymes, including phospholipase(Nilce *et al* ., 2021) .

3.6.4 Molecular detection o of heat shock protein *hsp90 gene* in *trichohyton species*

hsp90 gene was detected in *Trichophyton spp* isolates by using specific primers , it was found that 27 (100%) of *Trichophyton spp* isolated give the positive for this gene as shown in Table (3- 11) figure (3-5).

Table (3- 11) detection of *hsp90* in *trichohyton species*

Trichohyton species	Number N=27*
T. mentagrophytes	16/16
T. violaceum	4/4
T.rubrum	7/7
Total	27 (100%)

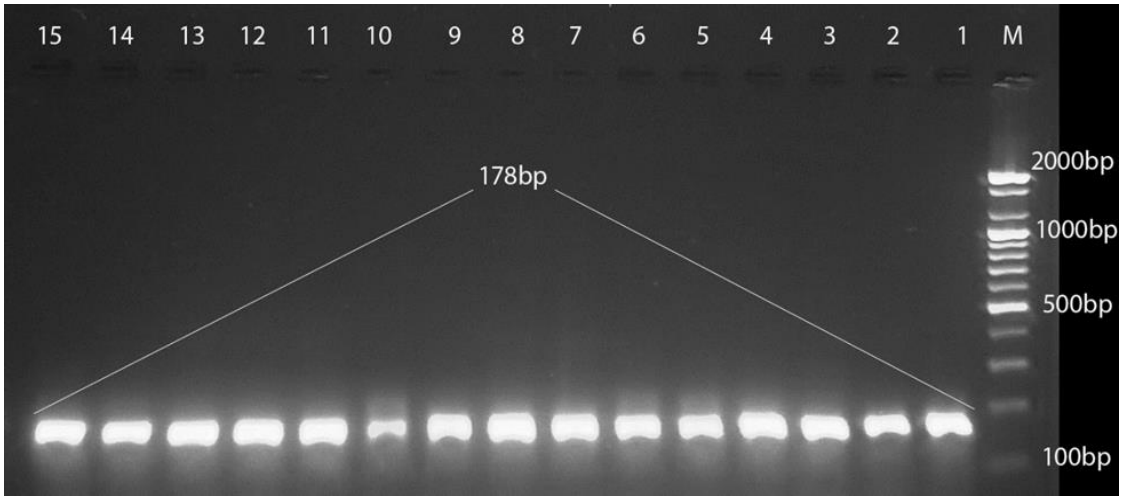


Fig. (3-5): Agarose gel electrophoresis of PCR product obtained with hsp90-specific primers that generated 178bp.. Lane M represent 100bp DNA ladder.lanes (1,2,3,4,5,6,9,11,12,13,14,15) were *Trichophyton mentagrophytes* ,lanes(8,10) were *Trichophyton rubrum* and lane (7) was *Trichophyton violaceum*. The electric current was allowed at 70 volt for 30 min , 1.5 % agarose.

In this investigation, detected a genetic virulence factors for *Trichophyton species* , This virulence factors was linked to an increased risk of skin and nail fungal infection . It is considered a rescue mechanism that enables the cells to cope under stressful conditions and protects from severe damage.

The regulation of heat shock proteins is a critical factor for promoting a quick and efficient response to several stressful conditions. Among the stimuli capable of triggering HSPs, highlight in dermatophytes those related to the infection of keratinized tissues (Martinez-Rossi *et al* ., 2017) .

It is one of the determining factors for the success of these organisms in colonizing host tissues is their ability to adjust their metabolism rapidly according to chemical and nutritional variations, a factor intimately associated with the presence of keratinolytic proteases and efficient response pathways to environmental stimuli (Martinez-Rossi *et al* ., 2011).

HSPs play an important role during the fungal infection of keratinized tissues, mainly through the effects of Hsp90 monitoring on signal transduction,

which regulates cell responses to environmental stimuli.When challenged with antifungal drugs, several dermatophytes generally react by activating stress responses , which often depends on the Hsp90 chaperone. Therefore, targeting Hsp90 or other related proteins may be a viable alternative to treat fungal infections caused by *T. rubrum* and probably by other dermatophytes as well. blocking the action of Hsp90 in dermatophytes becomes a potential strategy that combines this therapy with conventional antifungal drugs, which would enhance the overall outcome of the treatment (Leach *et al* ., 2012).

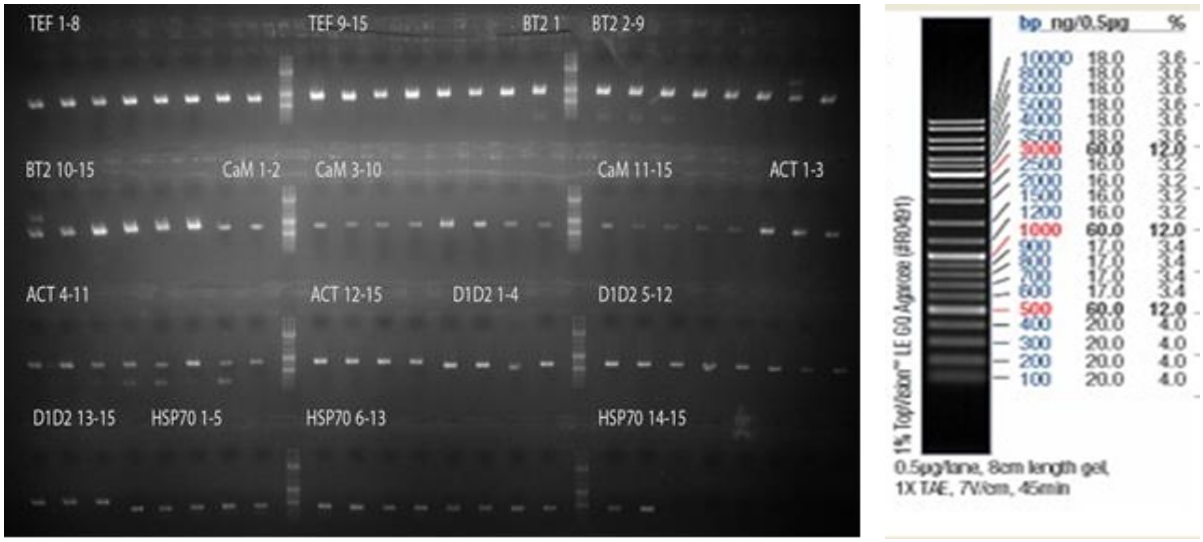
Hsps are conserved chaperones with multiple roles in the cell, such as aiding the folding and transport of proteins, protection under stressful conditions, and fungal pathogenicity (Nilce *et al* ., 2021) .

3.7 Multilocus sequencing typing of *Trichophyton species*

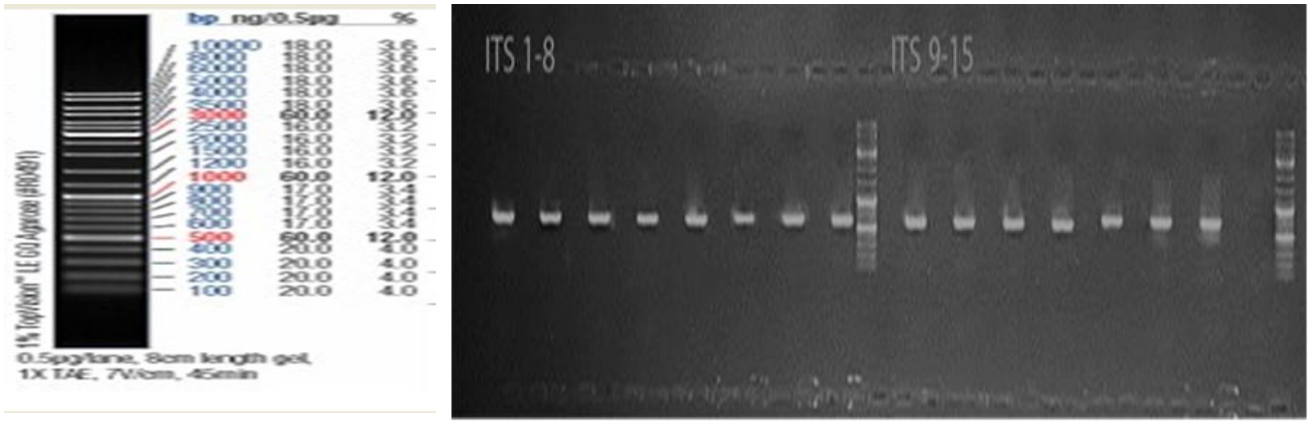
One of the valuable diagnostic techniques in molecular epidemiologic investigations is multilocus sequence typing (MLST) for the typing of multiple loci. In this technique, housekeeping genes along with other conserved genes are analyzed according to the nucleotide variation for the characterization of fungal pathogens (Chang *et al*., 2016).

The MLST was developed as a scalable typing system to determine the diversity and phylogenetic relationships of the isolates based on seven housekeeping genes, and it provide reproducibility, comparability, and transferability between laboratories.

The sequence data obtained for MLST for determining the population structures analyzing the extent of linkage disequilibrium between alleles for phylogenetic relationships between 15 isolates. Gel electrophoresis for each gene shown in figure (3-6 A – B) Table (3 -12 A- B)



Fig(3-6 A) :1% agarose gel electrophoresis for housekeeping genes for 15 *Trichophyton species* isolates. The electric current was allowed at 70 volt for 30 min



Fig(3-6 B) :1% agarose gel electrophoresis for housekeeping genes for 15 *Trichophyton species isolates.* The electric current was allowed at 70 volt for 30 min

Table (3-12 A) : housekeeping genes for 15 *Trichophyton species* isolates

	1	2	3	4	5	6	7	8	g	10	11	12
A	1-TR-1	11-TR-1	2-TR-2	12-TR-2	3-TR-3	16-TR-3	4-TR-4	17-TR-4	5-TR-7	18-TR-7	6-TR-5	19-TR-5
B	2-TR-1	12-TR-1	3-TR-2	16-TR-2	4-TR-3	17-TR-3	5-TR-4	18-TR-4	6-TR-7	19-TR-7	7-TR-5	20-TR-5
C	3-TR-1	16-TR-1	4-TR-2	17-TR-2	5-TR-3	18-TR-3	6-TR-4	19-TR-4	7-TR-7	20-TR-7	8-TR-5	
D	4-TR-1	17-TR-1	5-TR-2	18-TR-2	6-TR-3	19-TR-3	7-TR-4	20-TR-4	8-TR-7	1-TR-5	11-TR-5	
E	5-TR-1	18-TR-1	6-TR-2	19-TR-2	7-TR-3	20-TR-3	8-TR-4	1-TR-7	11-TR-7	2-TR-5	12-TR-5	
F	6-TR-1	19-TR-1	7-TR-2	20-TR-2	8-TR-3	1-TR-4	11-TR-4	2-TR-7	12-TR-7	3-TR-5	16-TR-5	
G	7-TR-1	20-TR-1	8-TR-2	1-TR-3	11-TR-3	2-TR-4	12-TR-4	3-TR-7	16-TR-7	4-TR-5	17-TR-5	
H	8-TR-1	1-TR-2	11-TR-2	2-TR-3	12-TR-3	3-TR-4	16-TR-4	4-TR-7	17-TR-7	5-TR-5	18-TR-5	

Table (3-12B) : housekeeping genes for 15 *Trichophyton species* isolates

	1	2
A	1-TR-6	11-TR-6
B	2-TR-6	12-TR-6
C	3-TR-6	16-TR-6
D	4-TR-6	17-TR-6
E	5-TR-6	18-TR-6
F	6-TR-6	19-TR-6
G	7-TR-6	20-TR-6
H	8-TR-6	

Identify each locus accurately , used sequence method. For all isolate , properly read and evaluated the seven genes by MLST ,polymorphic site ,GC content ,K (rate of nonsynonymous (dN) ,synonymous (dS) substitutions and the nucleotide diversity for each locus were determined as shown in table (3-13)

Table (3 - 13) Nucleotide and allelic diversity of the 7 genes evaluated

Locus	Size (bp)	Number of alleles	Polymorphic sites	GC content (%)	Nucleotide Diversity	*k*
ITS	668	5	112	57	0.04240	28.32381
BT2	767	9	123	51	0.03232	24.79048
TEF-1α	717	9	128	49	0.04660	33.40952
ACT	722	7	12	54	0.00435	3.14286
CaM	665	2	2	48	0.00040	0.26667
HSP70	399	12	90	48	0.05600	22.34286
D1/D2	582	10	90	57	0.02854	16.60952

k : Average number of nucleotide differences

The mean guanine cytocin content of sequnces of seven gene fragments ranged from 48%(*CaM* and *HSP70*) to 57 %(*ITS* and *D1/D2*):Trimmed fragment size of the 7 selected loci ranged from 399 bp (*HSP70*) - 767 bp (*BT2*).The nucleotide diversity ranging from 0.0004 to 0.05600 pergene . Moreover , The

number of polymorphic sites per locus varied between 2 (*CaM*) -128(*TEF-1 α*) . The polymorphic sites found in the present sequence data might therefore be useful in the development of a molecular fungal typing scheme based on detection of single nucleotide differences.

The proportion of nucleotide substitution that changed the amino acid sequence (nonsynonymous base substitution [dn] and the proportion that did note synonymous base substitution [ds]) were calculated ,and the dN/dS ratios which indicated negative selection were determined to be less than 1 for the *CaM* gene while were determined to be more than 1 for the *ITS , BT2 , TEF-1α, ACT , HSP70 and D1/D2* genes which indication positive selection.

A synonymous substitution is a nucleotide mutation which does not alter the amino acid sequence (silent mutation) while in a non-synonymous substitution, a nucleotide mutation manipulates the amino acid sequence of a protein and biological change in the organism.

The ratio (dn/ds) measure the level of selection in a protein coding gene. The ratio of dn/ds indicates purifying selection if dn/ds <1, positive selection if dn/ds >1. However ,the high ratios of nonsynonymous to synonymous substitution indict a role for diversitying selection these loci.

MLST protocol had sufficient discriminatory power to type isolates within a single species , it was analyze sequence diversity of seven housekeeping genes from are *Trichophyton species* isolates . Two loci had low polymorphisc (*ACT* and *CaM*) indicated that they had similar sequence at the species level . The *ITS* had 112 sites , *BT2* had 123 sites , *TEF-1α* had 128 sites , *HSP70* and *D1/D2* had 90 sites that suggesting recombination was evident and representing a significant source of genetic diversity of *Trichophyton species*. Fifteen *Trichophyton species* isolates were typed using MLST protocol.

In present study, MLST was used to explore the population structure and evolution of 15 *Trichophyton species* isolates from different clinical specimens which may provide better information concerning their biological properties. To initiate analysis ,the sequence diversity of the 7 housekeeping genes was calculate . This step was carried out to measure whether these selected loci had sufficient typing discrimination .

PCR of *ITS , BT2 , TEF-1α, ACT , HSP70 , D1/D2* and *CaM gene* in isolates showed bands size of which range from 399 to 767 bp .The *ITS* and *TEF-1α* sequence did not show any interspecies variation in all species as shown in appendix .

The characteristic of the multilocus obtained from the sequence of the PCR product from *T.mentaghrophytes* loci were compared to those of the corresponding loci in *T.rubrum* and *T.voliucosum* . The sequence were obtained from combination of seven genes which contained 4520 nucleotide .The HSP70 region was to be the shortest gene with 399 bp,while BT2 was the longest gene with 767 bp .

Isolates could be divided into 15 ST using combined data from 7 loci as shown in table (3 - 14). However all isolated samples were variable ST . The ST 1,2,3,4 were found in the leg ,ST 6,7,8 9 were found in back, ST 10,11,12 were found in abdominal 13,14 were found in chest and TS 5 ,15 were found in nail.

Table (3-14): Allele Profile of *Trichophyton spp* isolates showing allele number of seven loci and sequence type

Species	Site of infection	Isolates no.	ITS	*BT2*	*TEF-1α*	*ACT*	*CaM*	*HSP70*	D1/D2	ST
***T.* mentagrophytes**	**Skin (leg)**	**TR-1**	2	1	2	1	2	10	2	1
T. mentagrophytes	**Skin (leg)**	**TR-2**	2	8	2	2	1	11	3	2
T. mentagrophytes	**Skin (leg)**	**TR-3**	2	4	1	4	1	3	1	3
T. violaceum	**Skin (leg)**	**TR-4**	2	7	2	5	1	4	4	4
T. mentagrophytes	**Nail**	**TR-5**	2	4	3	1	1	9	5	5
T. mentagrophytes	**Skin(back)**	**TR-6**	2	5	2	7	1	8	4	6
T. mentagrophytes	**Skin(back)**	**TR-7**	3	2	7	2	1	7	4	7
T.rubrum	**Skin(back)**	**TR-8**	5	3	8	2	1	2	9	8
T. mentagrophytes	**Skin(back)**	**TR-11**	2	4	2	6	1	1	2	9
T.rubrum	**Skin (abdominal)**	**TR-12**	4	4	9	6	1	7	10	10
T. mentagrophytes	**Skin (abdominal)**	**TR-16**	1	6	4	6	1	6	5	11
T. mentagrophytes	**Skin (abdominal)**	**TR-17**	1	9	6	6	1	5	8	12
T. mentagrophytes	**Skin (chest)**	**TR-18**	1	7	4	2	1	5	7	13
T. mentagrophytes	**Skin (chest)**	**TR-19**	1	6	5	3	1	2	7	14
T. mentagrophytes	**Nail**	**TR-20**	1	6	4	2	1	12	6	15

In MLST different sequence at each locus are assigned with specific allelic profile and assigned as a sequence type which is the unambiguous descriptor of the strain .

This is the first study report describing the development and use of MLST to characterize this important human pathogen in Iraq. However, the result of this study present sequence type (ST1 to ST15) study done by (salehi *et la* ., 2021) when found that *T.rubrum* had 6 ST and *T mentaghrophytes* had 5 ST . It was also observed that with the three markers and two phylogenetic methods, the species *T.*

rubrum and *T. violaceum* showed molecular similarity. Zhan *et al* ., (2018) who showed that these two species were phenotypically different but highly similar phylogenetically, because their multilocus phylogeny (with five markers) and a comparison of their genomes show close affinity (Angélica *et al* .,2023) .However the variation between this study and other studies due to the limited number of isolates, or may be due to increased travel or undergoing natural accumulation of sequence variation in housekeeping genes.

Moreover, isolates obtained from diverse geographical locations, and during extended periods of time may give more genetic variability. Also, the present study ST were detected, these could be unique to the region, further analysis, possibly sequencing the gene of these strains could help to define the characteristics of these ST.

According to allelic profile it was found that the presence of variant (SNP, insertion, or deletion) between isolates. In the case HSP70 was more variant or mutant than other 6 housekeeping genes, contrary to the CaM which was the least variant. The gene chosen for the present MLST scheme seem to be representatives of the general polymorphism seen in house keeping genes of *Trichophyton species.*

The DNA sequence of each of the seven genes were analyzed by the maximum likehood method to be well suited for determination of phylogenetic relationships among *Trichophyton spp* isolates the housekeeping genes are assumed to be suitable for a population genetic study .

The sequence data obtained for MLST for determining the population structures analyzing the degree of linkage disequilibrium between alleles for phylogenetic relationships between 15 isolates as shown in and figure (3 – 7) from the seven housekeeping genes frequently used for MLST analysis of *Trichopyton*

species found in the tree constructed from the gene sequence

Fig. (3 – 7): Phylogenetic phylogram based on concatenated sequences of 7 loci from 15 *Trichophyton isolates* by maximum likelihood method. Different colors represent the different species

The DNA sequence were aligned and analyzed for each gene fragments. The phylogeny of these ST, an MLST phylogenetic tree of all the *Trichophyton species* strains was inferred maximum like hood approach from concatenated sequence. All *Trichophyton species* isolates showed polyphyletic lineage and revealed two distinct clusters, cluster A contain 13 isolates and cluster B contain two isolates .

The phylogenic relationship between fungal organism, potential problems associated with its ability to resolve the relationship between closely related species , due to an extremely low rate of natural mutation

The present finding provided strong evidence that *Trichophyton species* strains possess a high level of temporal stability and phylogeographical structuring,

supported largely by the phylogeographical signals observed in the phylogenic tree. However, the phylogeny tree for each gene was shown in appendix (1) .

The study was showed the genetic relationships between the *Trichophyton species* clones. The presence of dominant clone in the samples of hospital showed the presence of shared infection source among the patients.

The genetic diversity among these strains may be related to gene deletion, insertion, duplication or high rate of horizontal gene transfer mechanism.

Single locus phylogenetic tree were non congruent ,suggesting that recombination plays a role in the generation of diversity of *Trichophyton species* population.

The MLST was developed as a scalable typing system to determine the diversity and phylogenetic relationships of the isolates based on seven housekeeping genes, and it provide reproducibility, comparability, and transferability between laboratories.

Split decomposition analysis was performed on each locus separately and on the concatenated sequences of all ST ,as shown in the split graphs figure (3- 8).

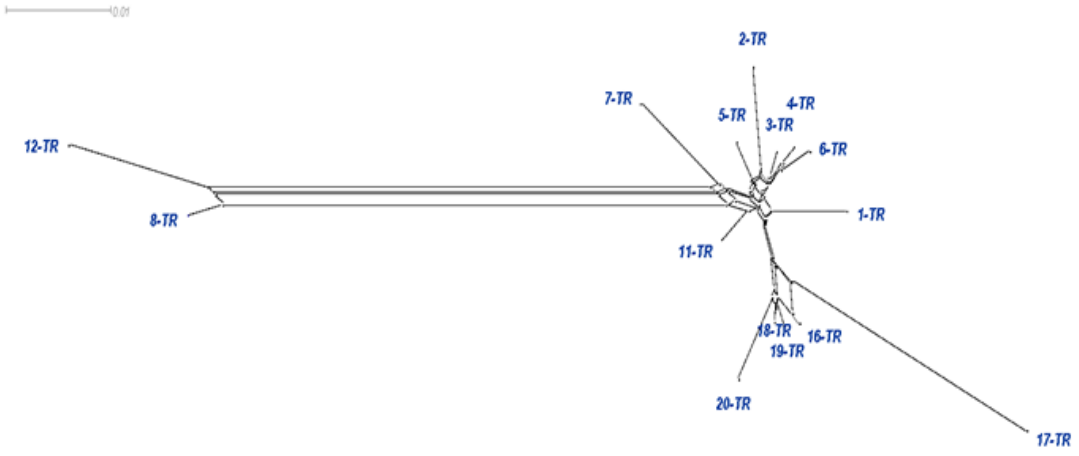
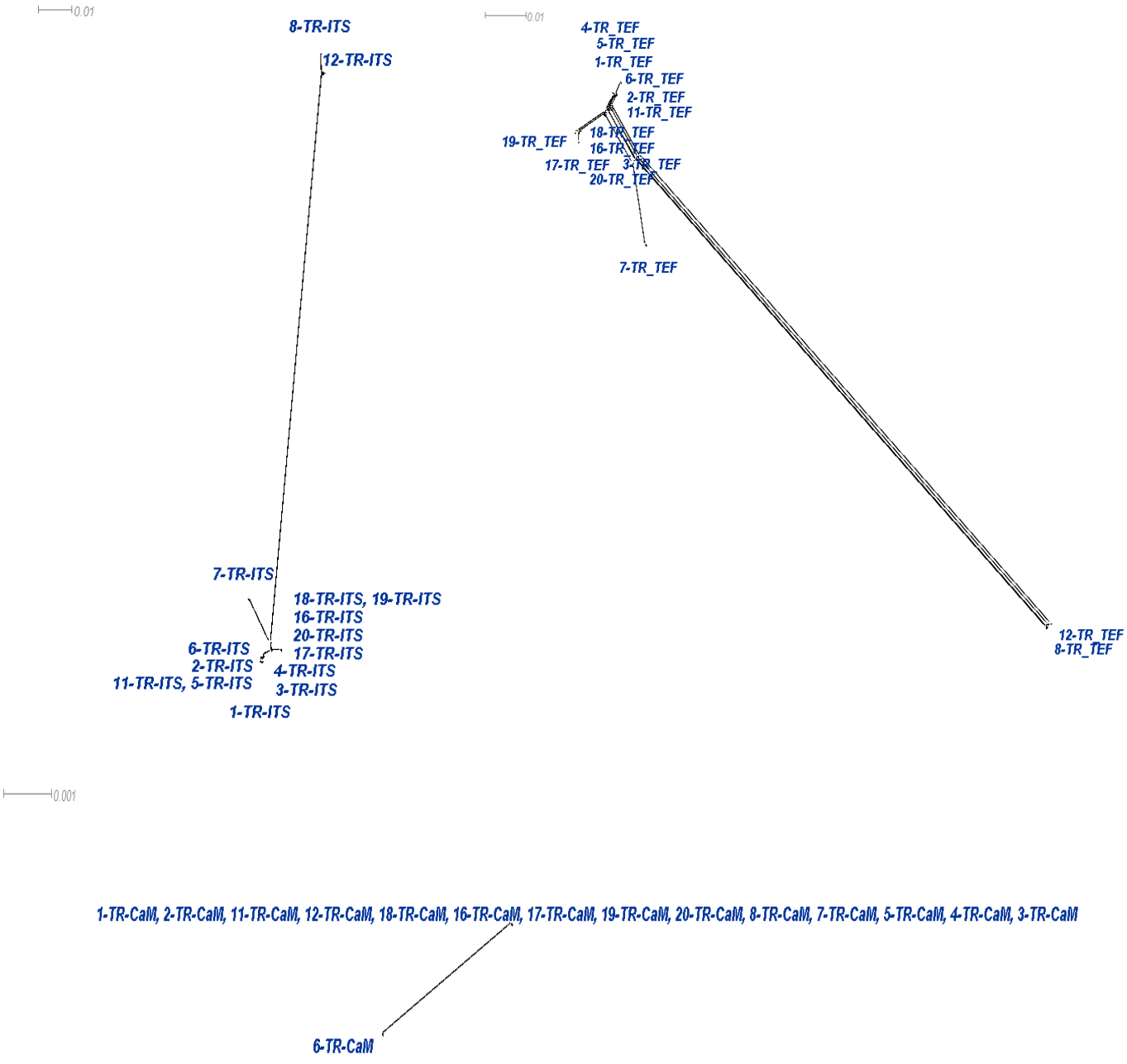


Fig. (3-8): Split-decomposition analysis based on concatenated sequences of 7 loci from 15 Trichophyton isolates. Note: multiparallelogram formations indicate recombination events

The split graphs for *ACT, BT2 , TEF, , CaM , HSP70* and *D1D2* revealed network like with parallelogram structures indicating that intragenic recombination had occurred during the evolutionary history of these genes . However ,the split graphs of ITS is tree like structures suggesting that the descent of these genes was clonal and absence of recombination . The split decomposition analysis of combined seven MLST Loci display network like structure with rays of different length as show in figure (3 -9).



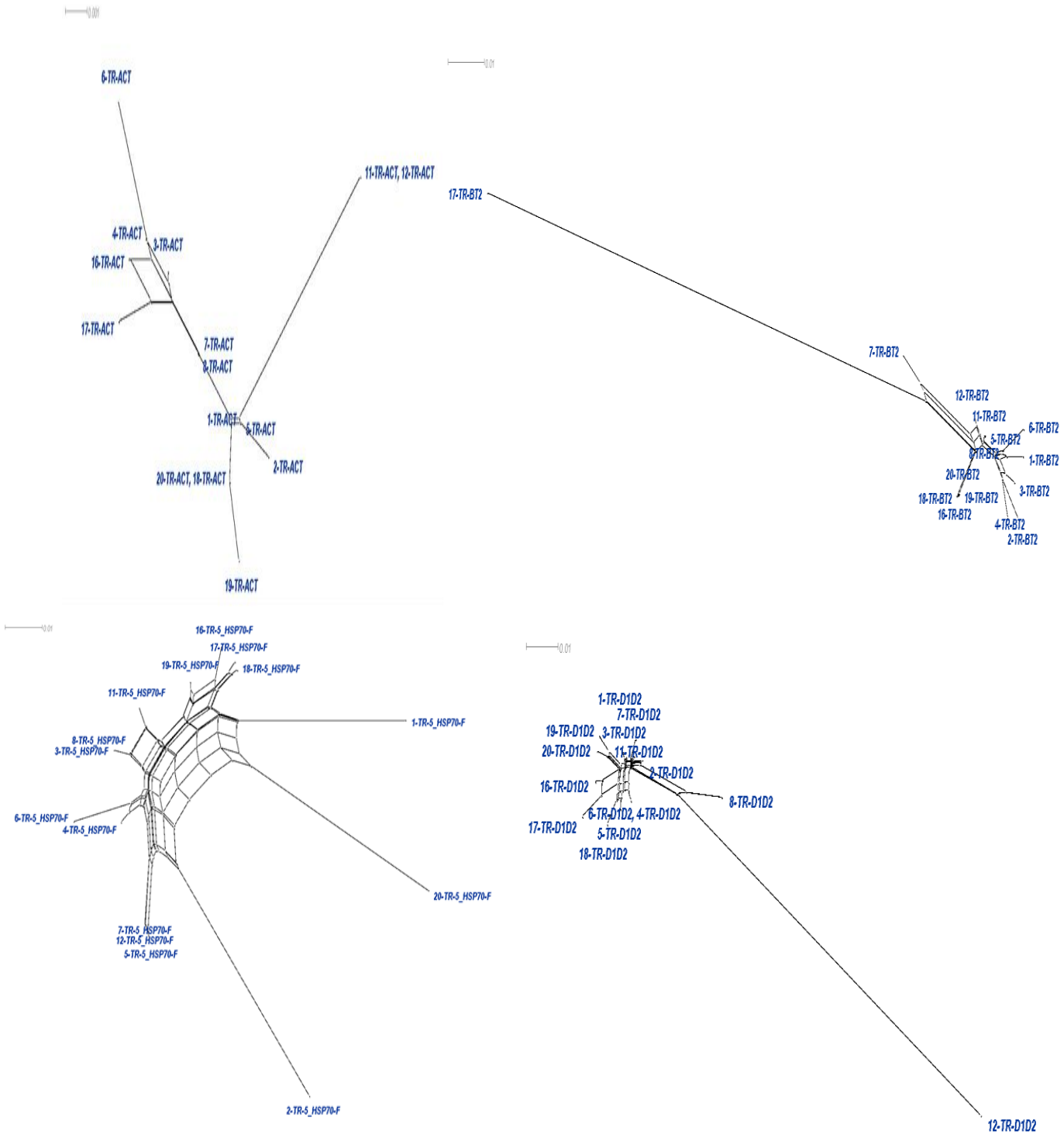


Fig. (3- 9): Split-decomposition analysis *ITS* , *BT2* , *TEF* ,*ACT* ,*CaM* , *HSP70* & *D1D2* gene sequences of 7 housekeeping genes from 15 *Trichophyton species* isolates. Note: multiparallelogram formations indicate recombination events

The 15 STs representing all isolates divided into subpopulation was completely disconnected.Split decomposition analysis based on the allelic profils of isolates have provided evidence of recombination that play a role in generating genotyping diversity among isolates.

In this study, tree like structures or parallelogram. Shaped structures were commonly found in the split graphs for all the seven housekeeping genes evaluated, illustrating that recombination had occurred in these MLST loci.

eBURST is an algorithm that can identifies groups of closely associated sequence types from MLST data. It was used to analyze the possible similarity ,variability and evolutionary relationships among different ST types of *trichophyon species* in the present study . The genetic backgrounds were found to be diverse among the STs identified in the present study. As shown in figure (3 -10) 4cc(cc1to cc4) were observed for the 15 ST. Among the 4 cc, cc4 was the largest and comprised 7 link ST, namely ST15, ST7, ST8, ST13, ST12, ST4 and ST11. These included two isolates from abdominal , two isolate from chest ,one isolate from nail and two isolate from back. Clonal complex 1, representing 5 ST ,which includes one isolate from back and abdominal , one isolate from nail , and two isolate from leg.While clonal complex 2 contain one isolates from each leg and back . Also cc3 contain one isolate from leg. Therefore ,it is unsurprising that the relationship between the isolates from clinical different source was closely related. Often, only some isolates of the same source or location were clustered to gather, whereas the rest were dispersed across other clusters .

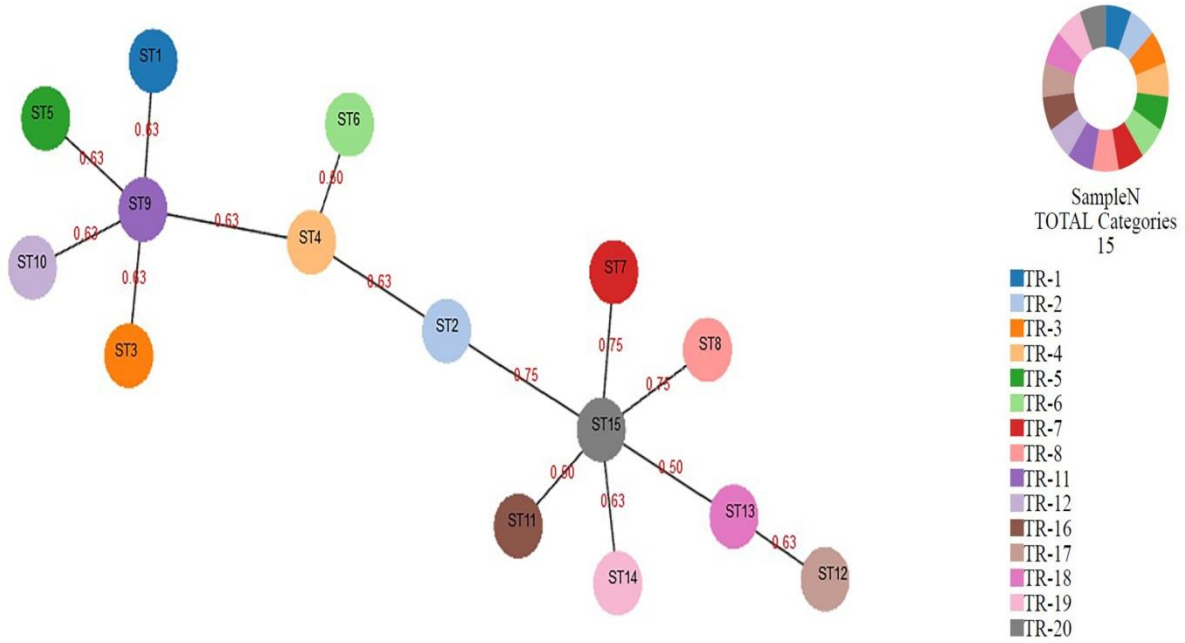


Fig (3 -10): Comparative eBURST analysis showing the clonal assignment and the relative distances of the identified STs

The eBURST analysis was performed for all species. Studies of this type are scarce and there is no database for MLST dermatophytes, thus, it was not feasible to compare the genotypes in the current study with those of the other studies. This study was a new starting point to determine the ST and foundation for a dermatophyte MLST database. The sequence of fragments of seven housekeeping genes from 15 of *Trichophyton species* isolates provide data that can be used to address aspects of the population and evolutionary biology of the species.

Trichophyton species frequent horizontal DNA exchange has eliminated the phylogenetic signal in each housekeeping gene. Determined the degree of allelic variations in seven housekeeping genes of *Trichophyton species* by using a sample of 15 isolates originated from different clinical specimens. The degree of isolates differentiation by MLST appear adequate for use in epidemiological investigation, as the number of different types obtained by MLST.

MLST was performed for only 15 isolates to determine the STs due to its high cost and labor intensive .

The high genetic variability amongst *Trichophyton spp* isolates in this study provides some information on the local dissemination and genetic relatedness.

Most previous studies showed that MLST was useful to accurately identify microorganism lineages, but few studies have considered the relationships between isolates and their source. In this study,used MLST to *Trichophyton species* isolated from different clinical specimens and look for relatedness to isolates that were pathogens. These representative isolates were unique in their diversity of sources and provide some necessary information required to understand genetic diversity persistence and movement in this species .

Trichophyton species housekeeping genes have similar but low nucleotide diversity values in general, reflacting that these genes are conserved .

Results of this study indicates that the majority of *Trichophyton species* studied may have descended from ancestor that exist many years ago. However, genetic variation in pathogen population is a major barrier to disease control .

MLST as a tool for epidemiological studies to investigate the evolutionary pathogen and clonal lineages of fungal . MLST differentiated strains into sequence of 7 housekeeping genes with appropriate level discrimination using allelic differences.

Although the number of isolates studied was not large, this work has shown that the specimens collected are highly divers. This is due either to a dynamic evolution of the local strain of the organism, or to the continuous introduction of new isolates from abroad. In this study provide valuable information that is

important for the understanding of the poor adaptation of *Trichophyton species* with uncommon STs, which may otherwise be capable of disseminating globally.

MLST is based on allelic variation in housekeeping genes, and while it monitors change over just a small portion of the genome, its highly discrimination and provides insight into genetic structure. Also, it reveals highly detailed information on genetic changes in specific housekeeping genes, and thus provides direct insight into evolutionary changes of the core genome. In addition, MLST proved useful for detection of novel and previously known strain and for inferring relatedness among isolates.

The current information will aid in the understanding of *Trichophyton species* infection global evolution and spread, as well as add to the existing data on this significant community-acquired infection that is a global public health concern. The results of this study contribute to our understanding of the global circulation of *Trichophyton species* sequence forms .

The data of this study have public-health implication, where the high diversity and emergence of a new clone of *Trichophyton species* in Iraqi province calls attention for the epidemic and the recognition of the strains with new clones that could protrude in the future, that in turn very important for understanding the evolution.

The dermatophyte species has been recently revised based on the MLST .For example, the recent study showed that *T. mentagrophytes* and *T. interdigitale* belong to the same phylogenetic species . There remain many uncertainties concerning the boundaries between dermatophytes species. Besides, to the best of the researchers' knowledge, there are a few studies with the MLST of dermatophytes .In recent years, the trend toward this technique has been increased to investigate dermatophyte species. Moreover, previous studies on MLST have

not addressed the genotypes, sequence types (STs) species, discriminatory power, ratio dN/dS, and informative sites (Zahra Salehi *et la* ., 2021) .

The multilocus sequence can distinguish between the individual strains and other isolates, and evaluate the relationships, recombination, and mutation rates among microorganisms, which are classified under the same genus (Zahra Salehi1 *et al* ., 2021)

Multilocus sequence typing (MLST) data has helped to clarify relationships of the major genera of *Trichophyton spp* resolved polyphyletic genera initially assigned by morphological or phenotypic data (Gabriela *et al* .,2018)

Generally, molecular typing methods are intended to tackle two different levels of epidemiological problems, which reflect different insights toward solving a local or global epidemiology in different time frames. In one hand localized outbreak of disease in a short period of time should be assessed and on the other, relation between strains causing a disease in one geographic area with those observed around the world during a longer period would be investigated. These two different conceptual views demand different appropriate scheme of molecular typing, so that isolates recorded in same molecular type are likely to be descended from a younger ancestor and those belonging to more distant ancestors are expected to differ in type unless a relative higher clonal population would be under study.

3.8 Whole genome sequencing

Whole genome sequencing and assembly were conducted by hybridizing next-generation sequencing (NGS) and using the Illumina MiSeq platform. Its gene annotation was extensively predicted using various BLAST databases, including non-redundant (Nr) protein sequence, Swiss-Prot, gene ontology (GO), kyoto encyclopedia of genes and genomes (KEGG) and eukaryotic orthologous groups (KOG), and carbohydrate-active enzyme (CAZy) databases.

At a genomic level, this study tried to discriminate between closely related strains, *Trichophyton mentagrophytes* (6 TR ,7 TR) isolates , by using whole genome sequencing depending on the next-generation sequencing method. According to this technique, two strains of *Trichophyton mentagrophytes* (6 TR ,7 TR) were subjected to whole genome sequencing. The resulted data were analyzed to calculate the general characteristics of *Trichophyton mentagrophytes* genomes, comparative genome analysis, gen annotation and variants calling.

The whole genome sequences of clinical isolates provided in this report can serve as a key reference point for a thorough and detailed investigation of clinical strains originating from different parts of the world and in devising disease management policies towards emerging resistance cases in Iraq. Genome sequencing has become one of the conventional means to study the biology and ecological abilities of microbes (Zhan *et al*.,2018).

3.8.1 General characteristics of *Trichophyton mentagrophytes* genomes

In the present study, whole-genome sequencing method generated a raw paired-end reads. For each genome, total number of bases sequenced, total number of nucleotides, GC content (%),genome size and contigs. Regarding the first raw data in *Trichophyton mentagrophytes* (6TR) , A 683.778614 M of total nucleotides bases were produced and total sequences were 4.531292 M. The GC content was 49.1% .*Trichophyton mentagrophytes* (7 TR) , A 2.443309 G of total nucleotides bases were produced and total sequences were 16.319284 M . The GC content was 47.57%.

To reduce bias in the data analysis, read trimming and filtering was performed using specific bioinformatics tools. After filtering, the same statistics criteria were calculated again. The analysis of the filtered raw sequences for (6TR) generated 678.139781 M of total nucleotides bases,

4.494508 M total sequences , 49.06% GC . Regarding (7TR) the results of data analysis were 2.423139 G of total nucleotides bases , 16.183000 M total sequences , 47.55% GC. After assembling, the final draft genome size of was 22146776 bp for *Trichophyton mentagrophytes* (6TR) and 22124453 bp for *Trichophyton mentagrophytes* (7 TR) . Additionally, the draft genomes of (6TR) and (7TR) contained on 540 and 908 contigs. Also ,the genome of (6TR) and (7TR) contained on 431982 bp and 389439 bp Max contigs , and Min contigs as 201 in (6TR) and 200 in (7TR) . General characteristics of *Trichophyton mentagrophytes* genomes are summarized in Table (3-15)

Table (3-15): General characteristics of *Trichophyton mentagrophytes* 6-TR and *Trichophyton mentagrophytes* 7-TR before and after filtration and assembly.

Characteristics		6TR	7TR
Before filtering	Total sequences	4.531292 M	16.319284 M
	Total nucleotides	683.778614 M	2.443309 G
	GC%	49.1	47.57
After filtering	Total sequences	4.494508 M	16.183000 M
	Total nucleotides	678.139781 M	2.423139 G
	GC%	49.06	47.55
Assembly	Genome Size	22146776 bp	22124453 bp
	Contigs	540	908
	Max contigs	431982 bp	389439 bp
	Min contigs	201	200

Regarding genome size, the results of this study noted very slightly differences among *Trichophyton mentagrophytes* genomes, where the genome of (6TR) was larger than (7TR). The variation in the size genome among

Trichophyton. *Mentagrophytes* isolates caused by extremely genomic rearrangements such as duplication and indels.

GC content present in the genome sequence of different isolate were predicate and this identifies that total GC content present in different isolates are different .

Genetic variation of an individual or variation within the species and between the species is better understood by whole genome sequencing process. An organism's genome sequence would represent the complete nucleotide base sequence for all chromosomes; this process is used for determining the ordered nucleotide sequences of entire genomes of organisms (Ekblom *et al* ., 2014).

Dermatophytes show an extraordinarily high coherence at nucleotide and gene level, with very few repeat elements in the genome. These data indicate that dermatophytes are consistent pathogens with a short divergence time ,g very few genetic events occurred in the evolutionary history of dermatophytes (Zhan *et al*.,2018).

3.8.2 Comparative genome analysis

The whole genomes of the studied *Trichophyton mentagrophytes* isolates (6 TR ,7 TR) were compared with each other or with the reference genome (TIMM 2789) to identify genomic rearrangements, conserved genomic regions and segmental gain or loss. This type of analysis is an attempt to understand how these genomes evolved and what are the main differences between them. At the whole-genome level, the patterns of Comparative genome analysis were performed, Multiple genome alignment comparison.

3.8.2.1 Multiple genome alignment comparison of *Trichophyton mentagrophytes* genomes

In this study, Multiple genome alignment comparison among the studied genomes of *Trichophyton mentagrophytes* and the reference genome was performed by using progressive Mauve. This type of comparative analysis identifies evolutionary events by aligning homologous regions of sequence resulting conserved segments, called locally collinear blocks (LCBs). Each LCB represents a homologous region of sequence conserved among all the genomes being aligned (Darling *et al*., 2010)

However, the results of this study revealed 1779 rearrangements distance based on pair wise LCBs among the aligned genomes Reference genome and the studied genomes of *Trichophyton mentagrophytes* (6TR) with different distance . On the other hand , this study revealed 1788 rearrangements distance based on pair wise LCBs among alignment genome , reference genome and the studied genome of *Trichophyton mentagrophytes* (7TR) with different distance. The presence of regions only in the studied strains may indicates to a pattern of segmental gain during the course of evolution. Moreover, the results of this study showed different patterns of genome rearrangement. In more details, there are several regions lack detectable sequence homology among the studied genomes. These regions represent sequences outside colored LCBs that visualized (marked as a thick arrow) in figure (3 -11). All the studied genomes of *Trichophyton. mentagrophytes* possessed regions with no sequence homology and the large region noted on *Trichophyton mentagrophytes*. The presence of this regions on the studied genomes may be related to frequent nucleotide substitutions that occurred by mutations.

The presence of these genetic segments refers to several evolutionally changes such as segmental insertion or deletion (indels) that could be related to the horizontal gene transfer. In addition, the genome of 6TR and 7 TR revealed more reciprocal LCBs, as illustrated on figure (3- 11) with thin arrow. These blocks are regions aligned in the reverse complement (inverse) orientation suggesting pattern of genomic rearrangements in 6TR and 7TR genome.

However , all the studied genomes of *Trichophyton mentagrophytes* (6TR , 7TR) showed different patterns of genomic rearrangements with each other or with the reference genome. Similarly, these genomes revealed conserved regions indicating the shared ancestry with the reference genome. In spite of this study may be the first study in Iraq that compared between *Trichophyton mentagrophytes* (6TR, 7TR)at whole genome level .

Globally, the evolution of epidemic strains of *Trichophyton mentagrophytes* has been significantly influenced by large-scale evolutionary events such as horizontal gene transfer, lateral gene transfer and genomic rearrangement such as inversions or relocations of DNA fragments, where observation of genome rearrangements between both closely related and divergent organisms represents a major force understanding evolution of *Trichophyton mentagrophytes*. For that reason, identification of genome rearrangements among *Trichophyton mentagrophytes* strains of Iraqi Province provided further insights into their history of evolution.

Comparative genomic analysis of dermatophytes identified if there is any specific change in any functional category that is common to all genomes of dermatophytes, which would help in suggesting the candidate genes having role in dermatophytosis and help in the new therapies development (Martinez *et al*., 2012).

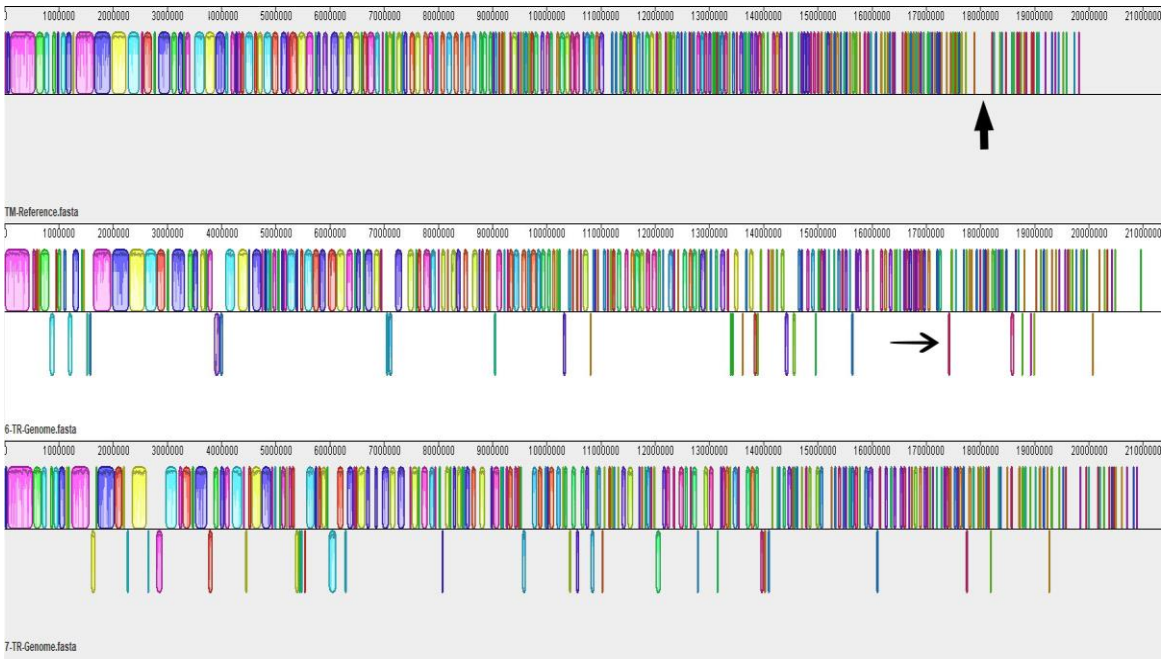


Fig. (3- 11): Comparative genome alignment of 6TR and 7TR samples in comparison to reference genomes (*Trichophyton mentagrophytes*). Sequence homology among genome contigs was assigned for each LCB with a unique color. Colored lines used to connect between similarly colored LCBs. Genomes from up to down are: reference genome of *Trichophyton mentagrophytes*, *Trichophyton mentagrophytes 6TR*, *Trichophyton mentagrophytes 7TR*. Symbols represent the following events: thin arrow=reciprocal LCBs; thick arrow=Sequences outside colored LCBs that do not have sequence homologs in the other genomes.

3.8.3 Annotation

In the present study, the whole genome sequences of the studied *Trichophyton mentagrophytes* (6 TR) and (7TR) were annotated to identify the coding regions (genes) in a genome, the locations of genes and its function. In addition, comparative proteome analysis was performed for each genome. According to genome annotation, the results of this study included general characteristics of genome annotation, proteome comparison, general predicted subsystem groups and identification of the specialty genes (Virulence factors genes).

3.8.3.1General characteristics of genome annotation for *Trichophyton mentagrophytes* strains (6TR ,7TR)

This study resulted several features regarding genome annotation of *Trichophyton mentagrophytes* strains (6TR , 7TR). These features included the number of genes , protein coding sequences (CDS), transfer RNA (tRNA) genes, ribosomal RNA (rRNA) genes intron , simple sequence repeat (SSR) and open reading frame (ORF) . Moreover, the function of these proteins was predicted for each genome. Accordingly, the results of (6TR) genome annotation showed that this genome has 24197 protein coding sequences (CDS), 93 transfer RNA (tRNA) genes, and 10 ribosomal RNA (rRNA) genes ,10658 SSR , 315607 ORF , 7851 genes and 16448 intron . The annotation included 461853 protein with functional assignments Table (3-16). The proteins with functional assignments included 18118 proteins with uniprot , 30515 protein with staring , 60760 protein with compartment , 312199 protein with gene ontology (GO) assignments, and 40261 proteins that were mapped to KEGG pathways. In contrast, (7TR) genome has different numbers, genome annotation showed has 24867 protein coding sequences (CDS), 82 transfer RNA (tRNA) genes, and 14 ribosomal RNA (rRNA) genes ,10722 SSR , 614839 ORF , 7768 genes and 17138 intron. The annotation included 453508 protein with functional assignments. The proteins with functional assignments included 17884 proteins with uniprot , 28596 protein with staring , 60041 protein with compartment , 307684 protein with gene ontology (GO) assignments, and 39303 proteins that were mapped to KEGG pathways. The annotated features are summarized in Table (3-16) .

Table (3-16): Annotated Genome Features of *Trichophyton mentagrophytes* 6-TR and *Trichophyton mentagrophytes* 7-TR

Features	6TR	7TR
Genes	7,851	7,768
Protein	8,177	8087
CDS	24197	24867
Intron	16448	17138
Repeat Regions	12,512	12,079
tRNA	93	82
rRNA	10	14
SSR	10,658	10,722
ORF	315,607	614,839
Number of pseudogenes	171	479
Number of non-coding genes	103	66
Functional features assignments	461853	453508
Proteins with GO assignments	312199	307684
Proteins with Pathway assignments	40261	39303
Proteins with UniProt assignments	18118	17884
Proteins with STRING assignments	30515	28596
Proteins with COMPARTMENTS assignments	60760	60041

According to above results, 7 TR strain resulted more CDS than 6TR. Moreover, genome of 7TR strain possessed more genes encoded for ORF and rRNA than other studied strains. This is small variety in numbers among the studied genomes could be related to the different abilities of gene gain or lose for each genome that effected by the selective pressure in the environmental conditions. Moreover, the higher number of pseudogenes were identified in 7TR strain rather than 6TR strain indicating to gain new genes with non-functions. These results may be good answer to interpret the predominance of *Trichophyton mentagrophytes* strain as a causative agent for skin disease disease in Iraqi Province .With regard to proteins with functional assignments in all studied strains, the results of this study revealed that most of these proteins were identified gene ontology protein more than other proteins that were mapped to KEGG pathways , protein with uniport or protein with compartments .Non coding repeat region such as simple sequence repeat (SSR) provide excellent information for assessing genomic variation and this frequency used as molecular marker for distinguish closely related isolates . In conclusion, alignment of genome sequences of the studied strains with these different resources of genome annotation data bases represent the extend of function variety among the studied strains .

3-8-3-2 Genome annotation of *Trichophyton mentagrophytes* (6TR ,7TR) genome

Conduct the functional annotation of the putative coding sequence in *Trichophyton mentagrophytes* (6TR ,7TR) were subjected to search function in gene ontology and kyoto encyclopedia of genes and genomes databases. Based on gene ontology assignment , were categorized into three main gene ontology categories as show in fig (3-12) .In term of biological processes which include metabolic processes , cellular processes. The cellular component was distributed across were the membrane part , cell part and organelles . while ,the molecular

function were involved catalytic (activity) binding , transport activity and transcription regulator activity .

Kyoto Encyclopedia of genes and genomes is the major public pathway related database .According to KEGG analysis , 9370 in (6TR) and 9099 in (7TR) were annoted and assigned to different KEGG which could be classified into six main KEGG categories : genetic information process , environmental informal processing , cellular processes , metabolism ,human diseases and organismal system as shown in figures (3-13) and (3-14)

Most annotated gene were attributed to metabolism pathways , and the associated pathways primarily contained amino acid metabolism ,carbohydrates metabolism and energy metabolism .

Both GO and KEGG annotation were mainly concentrated in the basic metabolism and host infractions on with *Trichophyton mentagrophytes* depends.

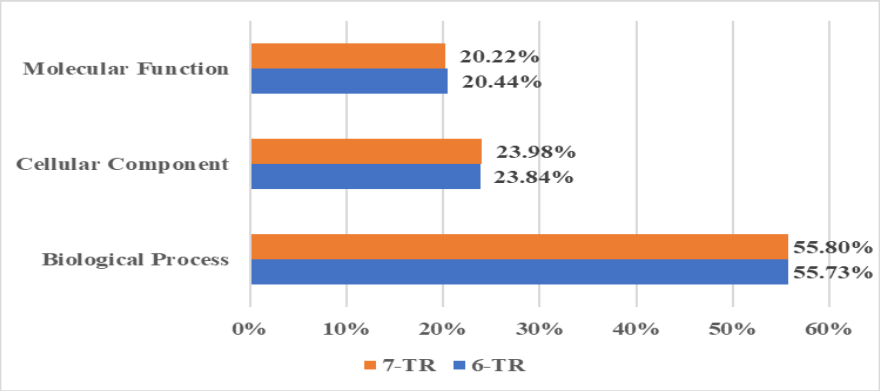


Fig (3-12) Genome annotation features according to Gene Ontology assignments

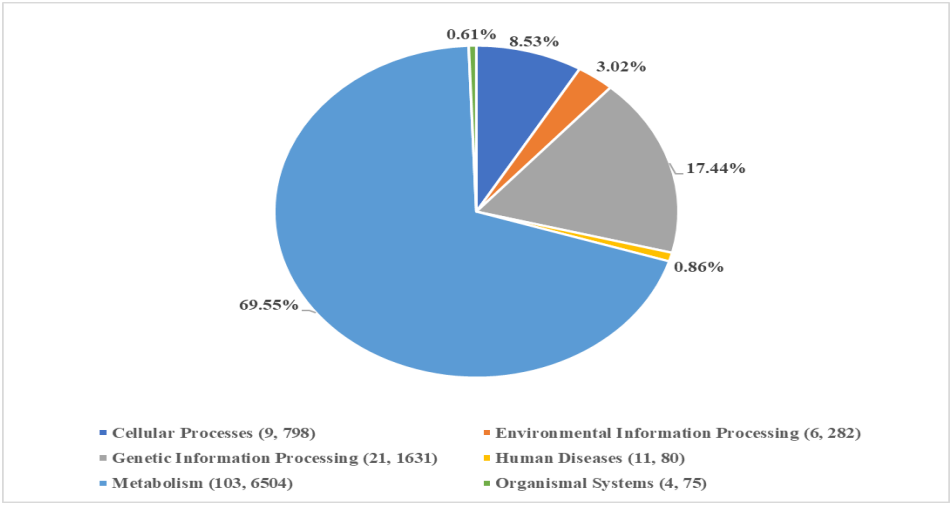


Fig (3-13) Genome annotation features percentages for 6TR isolate according to KEGG pathway Classification (pathway, genes).

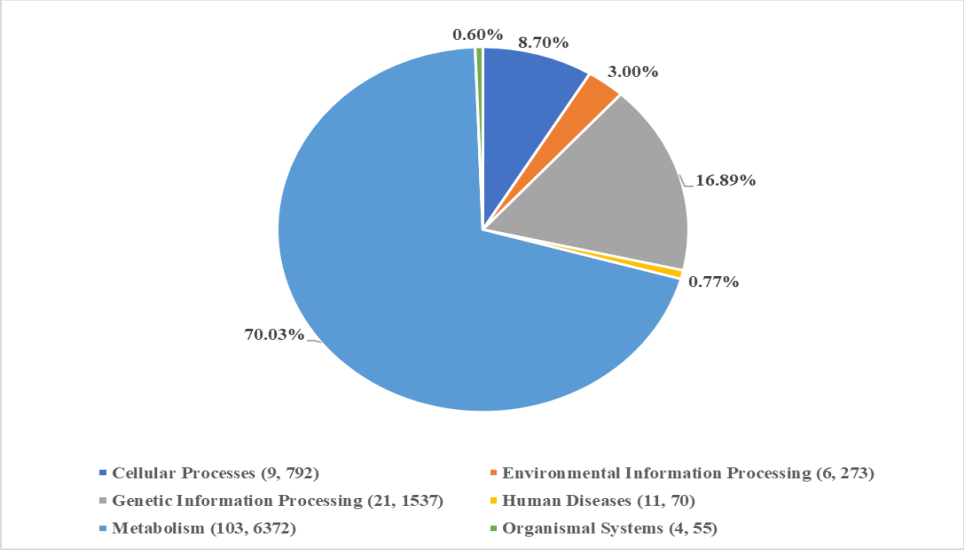


Fig (3-14) Genome annotation features percentages for 7TR isolate according to KEGG pathway Classification (pathway, genes).

3.8.3.3 Annotation of the Virulence Factor genes across the genomes of *Trichophyton mentagrophytes* (6TR,7TR)

Virulence factors might contribute to the pathogenicity and host restriction of *Trichophyton* , but the underlying mechanism is not yet fully understood. Although the genome sequences of several host specific and broad-host *Trichophyton* are available.

Our study identifies families of genes that are likely involved in the interactions between dermatophytes and their hosts. These gene families may help to provide explanations for long-standing questionsin dermatophyte pathogenesis.

In this study, the main Virulence Factors involved in the pathogenesis of *Trichophyton mentagrophytes* were identified across the whole genome of studied *Trichophyton mentagrophytes* (6TR and 7TR). This type of specialty genes and protein was determined as Appendix (2) and (3)

Hypothesized that the level of virulence , as measured by the *Trichophyton* burden in cells and tissues using different models of infection may be predicted as high or low when combining WGS and phylogeny studies for the presence or absence of gene association with virulence.

The availability of sequence information allow research to use bioinformation approach to make predication about which genes are involves in virulence , as well as differences between isolates that have adapted to majority of which are annoted . This is not surprising and confirms the genetic relatedness of *Trichophyton*. However ,there are a number of hypothetical genes unique to each isolate that potentially play role in niche adaptation and pathogenicity .

This comparative genomic analysis of different *T.mentarophytes* strains help in identification of unique gene sequence features and gene families that are present in dermatophyte species that may cause fungal infection.

This study identifies that these dermatophyte strains of *T.mentagrophytes* have number of genes that are coding for lipases, esterases and other related proteins that are known to be lipid degradation enzymes. All two genomes of *T.mentagrophyetes* strains were found to encode large numbers of peptidases-encoding genes. Different number of genes in all strains encoding for aspartic

peptidases, cysteine peptidases, glutamic peptidases, metallo peptidases, asparagines peptidases and serine peptidases.

The availability of genome sequences of different dermatophytes help in identification of unique gene sequence features present in dermatophyte species that may cause fungal infection. Identification and characterization of virulence associated genes, genes involved in secondary metabolite biosynthesis and secretory derivative enzymes in dermatophytes may help in development of new therapies, this may help in understanding host dermatophyte interaction and role of these virulence related genes in pathogenicity of dermatophytes. This could and also help in finding the reasons for drug resistance in dermatophyte species that prevents dermatophyte species from complete clearance and result in treatment failure.

Different gene families in dermatophytes are responsible for infection, dermatophytes secretes different proteases (subtilisin protease,keratinases , lipases, kinases and other gene families (carbohydrate active enzymes) that are responsible for keratinized tissue degradation.

Presence of virulence genes encoded in the genomes of dermatophytes enables them to establish and maintain infection and survive on the outer cornified layers of skin of the host. A comparative analysis of virulence factors in the genomes using online prediction tools can reveal genomic features related to high pathogenicity of strains belonging to different genotypes and different geographical locations. Secreted lipases and proteases are other important enzymes that not only serve as key virulence factors but also aid the dermatophytic fungi in surviving on the terminally differentiated keratinized layers of skin by deriving nutrients from it (Martinez *et al* ., 2012).

Dermatophytes also encode a large number of proteases in their genomes، with a key role of secretory subtilases in adherence and in initial stages of infection in the host cells.

Protease may be used during specific stage infection or might have a more general role in growth . Furthermore , it is possible that the *Trichophyton* will have a unique program of expression for proteases and other putative virulence factors during infection . Secreted proteases have earlier been suggested to be important virulence factors of dermatophytes and found to be enriched in dermatophyte genomes (Martinez *et al*., 2012) .

Dermatophytes hence encode a wide repertoire of redundant secreted proteases in their genomes, which would be critical to its ability to utilize keratin as the sole source of carbon and nitrogen during superficial infections. Indeed, many subtilisin-like proteases of *T. rubrum*, namely, Sub3 and Sub5 on keratin or Sub3 and Sub4 in media containing human skin sections are upregulated during growth . Sub3, Sub4 and Sub7were also found to be upregulated during growth on keratin in another closely related dermatophyte, Arthroderma benhamiae while Sub1, Sub2, Sub3, Sub6 and Sub7 were identified in the secretome of A. benhamiae in a guinea pig infection model (Chitra *et al* ., 2015) .

3.8.3.4 Identification of carbohydrate active enzyme (CAZy) families in *Trihophyton mentagrophytes* (6TR ,7TR) genome

Carbohydrate active enzyme (CAZy) families are divided in to five classes , glycoside hydrolysis (GHs), carbohydrate estrases (CEs) ,glycotransferases (GTs) , Auxiliary activities (AA) and carbohydrate binding module (CBM) .

In the present study ,the genome sequence of *Trichophyton mentagrophytes* (6TR ,7TR) revealed several gene associated with assembly (GT) and break down (GHs ,CEs) of carbohydrate complex . In addition *Trichophyton mentagrophytes* (6TR ,7TR) genome was found to contain a vast array of genes coding for initial degradation auxiliary activity (AA) , as well as a carbohydrate binding module (CBM) .Annotation of the predicated amino acid sequence using CAZy data base of *Trichophyton mentagrophytes* (6TR ,7TR) related 46 % GHs ,42 % GT , 6% AA ,5 % CBM and 1% CEs in the *Trichophyton mentagrophytes* genome as show in figure (3-15) and (3-16)

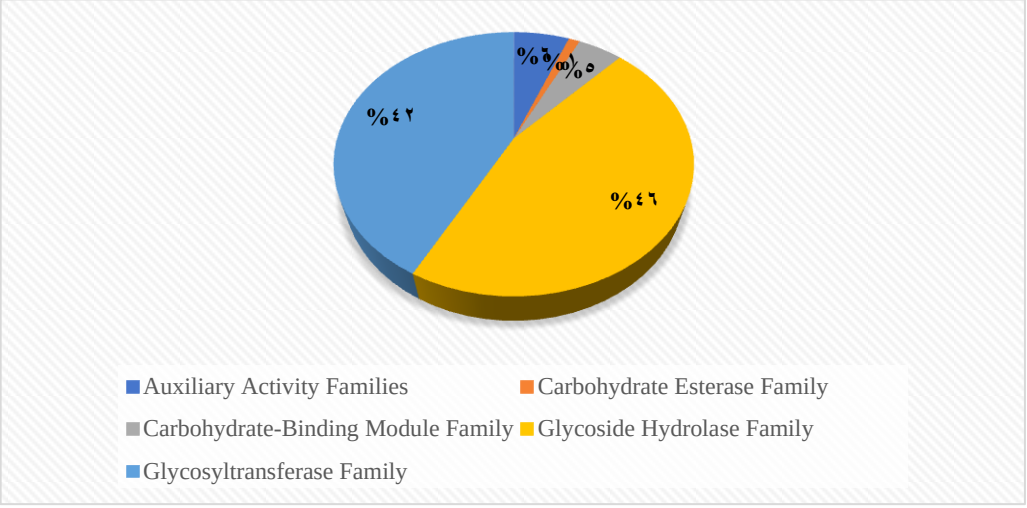


Fig (3-15): predication of Carbohydrate-Active enZYmes (CAZy) families in 6TR genome.

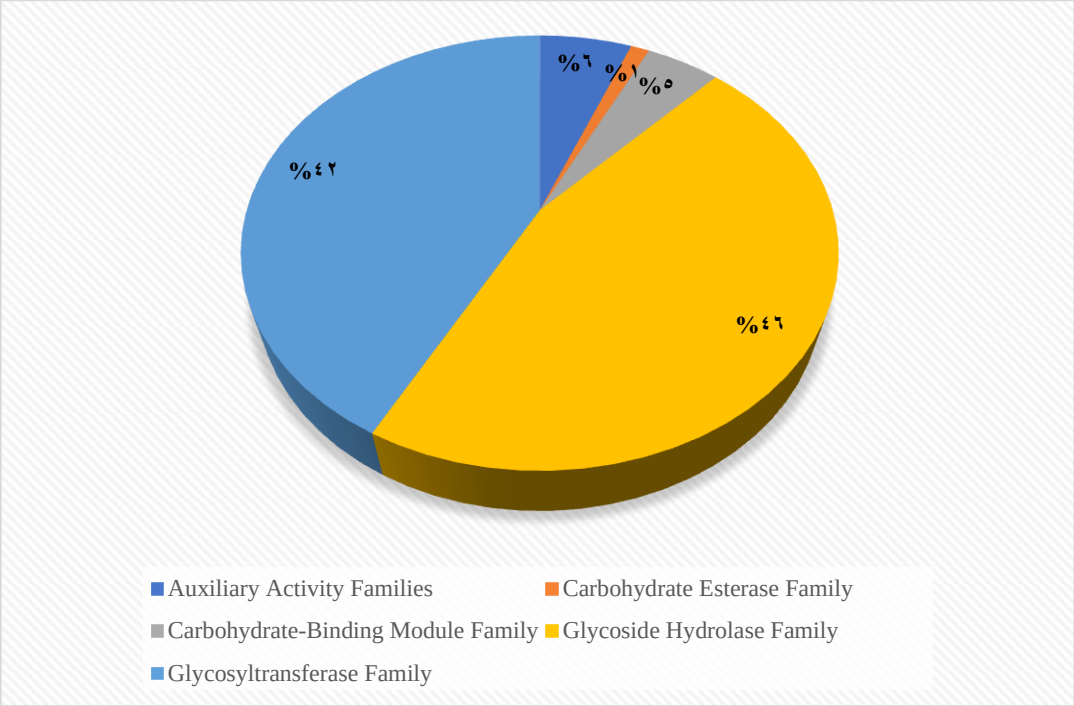


Fig (3-16): predication of Carbohydrate-Active enZYmes (CAZy) families in 7TR genome.

The CAZy family is the important role of protein degradation in the life style of dermatophytes.

Analysis of various carbohydrate-active-enzymes (Auxiliary activities, Carbohydrate binding modules, Carbohydrate esterases, Glycoside hydrolases ,Glycosyl transferase and polysaccharide lyases (PL)) shows different number of CAZymes are present in different strains of *T.mentagrophytes* responsible for hydrolysis of carbohydrates present in skin. It has been found that no gene is coding for Polysaccharide lyases responsible for non-hydrolytic cleavage of glycosidic bond .

Glycosyltransferases are enzymes that catalyze the formation of glycosidic linkages to form glycosides, which are involved in the biosynthesis of oligosaccharides, polysaccharides, and glycoconjugates . These enzymes utilize activated donor sugar phosphates and catalyze glycosyl group transfer to specific acceptor molecules to form glycosidic bonds (Lairson *et al* ., 2008) .

GTs are resident membrane proteins of the endoplasmic reticulum and golgi apparatus. All GT proteins have large C-terminal catalytic domains, a short N-terminal cytoplasmic tail, and a signal-anchor domain (16–20 amino acids). Signal-anchor domains act as both transmembrane regions and un cleavable signal peptides (Young *et al* ., 2018) .

Previous studies have described the difficulty of identification and classification of GTs based on sequence similarity; therefore, a GT identification method that does not rely solely on sequence similarity is required, for example, the development of a computational method to identify the transmembrane region of Golgi-localized signal-anchor-type GTs and discover novel GTs (Mukai *et al* ., 2008).

Glycoside Hydrolases (GHs) are enzymes that catalyze the hydrolysis of glycosidic bonds of complex carbohydrates and key enzymes involved in carbohydrate metabolism. In addition, GHs are common enzymes in nature that degrade the most abundant biomasses, such as cellulose, hemicellulose, and starch. GHs can be assigned to various families using algorithmic methods based on sequence similarity. Henrissat conducted comparisons of 301 amino acid sequences of GHs and classified 291 sequences into 35 families (Berlemont *et al* ., 2016) .

Fungi play an important role in the hydrolysis of cellulose, xylan, and chitin in the environment and thus have potential uses in biotechnology.

Carbohydrate-Binding Modules (CBMs) : Amino acid sequences having carbohydrate-binding activity within a carbohydrate-active enzyme are designated CBMs, which fold into structurally discrete modules . Generally, CBMs bind to carbohydrate ligands and enhance the catalytic efficiency of carbohydrate-active enzymes .

CBMs are most commonly associated with GHs. They have also been found in several PLs and GTs . In addition, CBMs present in proteins without hydrolytic activity are parts of a scaffolding (scaffoldin) subunit that organizes the catalytic subunits into a non-covalent multi-protein complex called a cellulosome . Enzymatic complexes bearing CBMs show more efficient degradation of substrates, and catalytic efficiency is reduced when CBMs are removed from the scaffolding of cellulosomes (Shoseyov *et al* ., 2006) .

Similar to glycoside hydrolases, CBMs can be classified into families based on amino acid sequence similarity. Until recently, CBMs have been classified into 80 families with more than 127,000 classified and 500 non-classified CBM sequences in the CAZy database (CAZy database; http://www.cazy.org) .

Carbohydrate Esterases (CEs) Esterases, which act on ester bonds, are widely used as biocatalysts in industrial processes and biotechnology. CEs represent a class of esterases that generally catalyze *O*-de- or *N*-deacylation to remove esters of substituted saccharides . These CEs are classified into 15 families, with more than 54,900 classified and 1200 non-classified CE sequences in the current CAZy database (CAZy database; **http://www.cazy.org**). CEs show great diversity in substrate specificity, such as specificity for xylan (acetylxylan esterases, EC 3.1.1.72), acetic ester (acetyl esterases, EC_3.1.1.6), chitin (chitin deacetylases, EC 3.5.1.41), peptidoglycan (poly-*N*-acetylglucosamine deacetylases, EC 3.5.1.104), feruloyl-polysaccharide (feruloyl esterases, EC 3.1.1.73), and pectin (pectinesterase, EC 3.1.1.11) (Biely , 2012) .

Finally ,auxiliary Activities (AAs) Members of families GH61 and CBM33 were found to be lytic polysaccharide monooxygenases (LMPOs), resulting in reclassification of these families into a new category in the CAZy database .Lignin degradation enzymes such as LMPOs are classified into AA families in the CAZy

database, and members of these families are mainly involved in depolymerization of non-carbohydrate structural components (lignin) or found as primary cell wall contents of plants . These AAs are classified into 15 families, with more than 10,300 classified and 100 non-classified AA sequences in the current CAZy database (CAZy database; http://www.cazy.org) (Young *et al* ., 2018) .

3.8.3.5 Analysis of secondary metabolite biosynthetic gene cluster

Trichophytons are enriched for genes connected to secondary metabolic production .The whole genome sequence of *Trichophyton mentagrophytes* (6TR ,7TR)were submitted to the anti SMASH (Antibiotics and Secondary Metabolite Analysis Shell) database for secondary metabolite BGCs analysis .Anti SMASH database analysis demonstrates that *Trichophyton mentagrophytes* (6TR) possessed 22 secondary metabolic BGCs ,including 2 terpene ,1 hybrid (terpene ,T1PKS),2 hybrid (NRPS ,T1PKS), 4 T1PKS ,4NRPS- Like , 6 NRPS and 1 for each indole , fungal –PiPP-like and betalactone . While in *Trichophyton mentagrophytes* (7TR) , the anti SMASH analysis demonstrated that possessed 24 secondary metabolic BGCs , including 2 terpene , 1 hybrid (T1PKS,NRPS-like),1 hybrid (NRPS ,T1PKS),1 hybrid (T1PK ,indol).6 T1PKS ,6 NRPS- Like , 4 NRPS and 1 for each indole , fungal –PiPP-like and betalactone . However , hybrid (T1PKs,NRPS-like) and hybrid (T1PK ,indol) absent in 6TR isolate .While , hybrid (terpene ,T1PKS) was absent in 7TR isolate as show in figure (3-17)

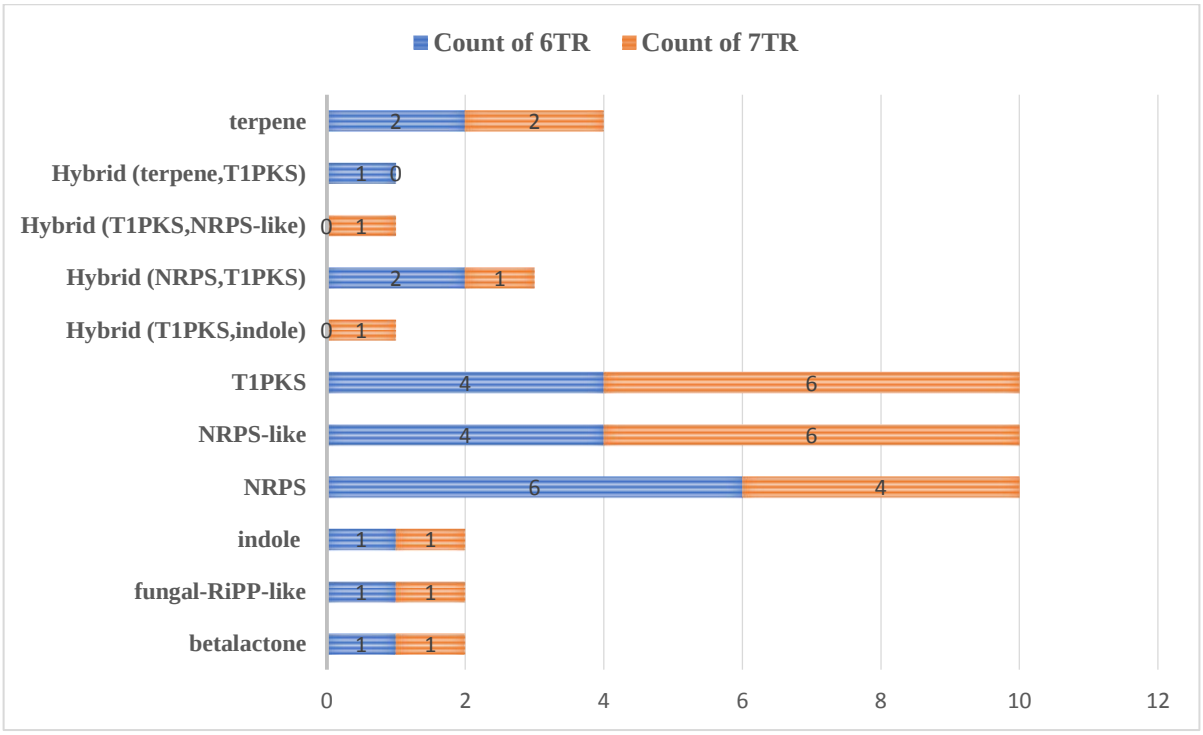


Figure (3-17) AntiSmash analysis for identification, annotation and analysis of secondary metabolite biosynthesis gene clusters (BGCs) for both studied Isolates 6TR and 7TR. NRPS; Non-ribosomal peptide synthetase, NRPS-like NRPS-like fragment, T1PKS; Type I PKS (Polyketide synthase), RiPP; ribosomally synthesised and post-translationally modified peptide product.

Most of BGCs in *Trichophyton mentagrophytes* (6TR,7TR) were unknown and yet to be unveiled .In (6TR) isolate , only 9 of these BGCs (40.9%)which include 1NRPS ,1NRPS-T1PKS, 2NPRS –like, 3 T1PKs , 1terpene and 1 terpene , T1PKS biosynthetic genes show similarity to know gene cluster in database as shown in table (3-19). Therefore , anti SMASH analysis display 22 secondary metabolite BGCs in 6TR isolate and only 9 of them show similarity with known gene cluster indicating the other 13 BGCs are unknown and yet to unveiled .

By further comparison , two BGCs with 100% similarity to the gene sequences on the other reference strain were identified and predicated to be responsible for biosynthesis of FR901512(polyketide) and clavaric acid , neosartoricin B polyketide with 60 % similarity . The other six BGCs show low similarity < 50 % to the gene sequence of the reference strain ,such as one NRPS

BGCs displayed 18% similarity with BGCs for pencillin and other similarity of BGCs Was found in table (3-19)

Table (3-19): AntiSmash analysis for identification, annotation and analysis of secondary metabolite biosynthesis gene clusters for both studied Isolates 6TR

6TR	Most similar Cluster	Similarity
betalactone		
fungal-RiPP-like		
indole		
NRPS		
NRPS		
NRPS	Penicillin	18%
NRPS		
NRPS		
NRPS		
NRPS,T1PKS	pyranoviolin A Polyketide+NRP	25
NRPS-like		
NRPS-like		
NRPS-like	FR901483 (NRP)	16%
NRPS-like	hexadehydroastechrome/terezine-D/astechrome	37%
T1PKS		
T1PKS	6-MSA/aculin A Polyketide	28
T1PKS	FR901512 (Polyketide)	100%
T1PKS	Waikikiamide /semivioxanthin Polyketide	45%
T1PKS,NRPS		

terpene	clavaric acid	100%
terpene		
terpene,T1PKS	neosartoricin B Polyketide	60%

On the other hand ,(7TR) isolate ,only 9 of these BGCs (37.5%)which includes 1 indole ,1NRPS , 3T1PKS ,1T1PKS- indole, 1T1PKS -NPRS ,1T1PKST NPRS –like and 1terpene biosynthetic genes show similarity to know gene cluster in database as shown in table (3-20) . Therefore ,anti SMASH analysis 24 secondary metabolite in BGCs in (7TR) isolate and only 9 of them show similarity with known gene cluster indicating the other 16 BGC are unknown and yet to unveiled .

By further comparison , low BGCs with 100% similarity to the gene sequence of other reference strain were identified and predicted to the responsible for biosynthesis of xenolozoyenone and clavaric acid , neosartoricin B polyketid with 80% similarity and swainonine with 66% similarity . The other five BGCs show similarity < 50% to the gene sequences of the reference strains , such as one NRPS BGCs displayed 12 % similarity with BGCs for penicillin and the other similarity BGCs was found in table (3-20).

Table (3-20): AntiSmash analysis for identification, annotation and analysis of secondary metabolite biosynthesis gene clusters for both studied Isolates 7TR

7TR	Most similar Cluster	Similarity
Betalactone		
fungal-RiPP-like		
indole	fumigaclavine C (Alkaloid)	36%
NRPS	penicillin (NRP)	12%
NRPS		

NRPS		
NRPS		
NRPS-like		
NRPS-like		
NRPS-like		
NRPS-like		
NRPS-like		
NRPS-like		
T1PKS		
T1PKS	waikikiamide-semivioxanthin/ Polyketide	45%
T1PKS	yanuthone D (Polyketide)	20%
T1PKS		
T1PKS		
T1PKS	equisetin (NRP+Polyketide)	18%
T1PKS,indole	neosartoricin B (Polyketide)	80%
T1PKS,NRPS	xenolozoyenone (NRP:Lipopeptide+Polyketide:Iterative type I polyketide)	100%
T1PKS,NRPS-like	swainsonine (NRP+Polyketide)	66%
terpene		
terpene	clavaric acid (Terpene)	100%

Secondary metabolites that are produced by these dermatophyte species are also responsible for infection .Secondary metabolites are a remarkably diverse class of cellular products that often exhibit taxonomic specificity. On the other hand , generally considered "nonessential" for organismal growth in culture . It has

long been known that dermatophytes can survive in toxic environments, presumably through the production of biological tools to resist these toxins (Lingling *et al* ., 2006) .

The gene sequence of *Trichophyton mentagrophytes* will prove to be invarious metabolic pathway along with elucidation of molecular mechanism of it pathogenesis and in comparative analysis of *Trichophyton mentagrophytes* isolates affecting other parts of the world .

Similar to other pathogenic filamentous fungi, such as *Magnaorthe grisea* and *Aspergillus fumigatus* , several important secondary metabolic products, were also found in *T. mentagrophytes* ; these included non-ribosomal peptide synthases, polyketide synthases, two putative dimethylallyl tryptophan synthases, a putative arsenate reductase and a hydrophobin. These gene products may be related to *T. mentagrophytes* growth and pathogenicity. Polyketides (derived from polyketones) are a class of secondary metabolites produced by most organisms, but they have been most extensively examined in bacteria and fungi. In fungi, numerous functions have been proposed for polyketides, including the production of toxins and spore pigments (Lingling *et al* ., 2006) .

Secondary metabolites (SMs) play important roles in the pathogenesis of microorganisms. In pathogenic fungi, polyketide and nonribosomal peptides have been shown to be the potential virulence factors and immunosuppressant. Identification of these factors is therefore highly important towards understanding the molecular basis of host-pathogen interactions. Genome sequencing of numerous dermatophytes belonging to the *Trichophyton* genera revealed that each genome encodes numerous SM clusters. Unfortunately, under laboratory conditions many of the gene clusters in fungi are silent and hence mask the products encoded in them .The difficulties involved in manipulating these fungi,

such as long doubling times and lack of genetic tools, further impedes the establishment of metabolite-cluster correlations. Neosartoricin was shown to inhibit T-cell proliferation and was proposed to possibly play a role in suppressing host adaptive immune response during infection (Wen *et al* .,2013) .

More recent expansion of secondary-metabolite enzymes in the dermatophytes suggests that they require an extensive arsenal of antimicrobial or toxic compounds. This arsenal may be necessary to survive in the various niches where dermatophytes deal with different environmental stresses (soil, skin, hair) with different hosts (plants and animals), and with different cohabitating microbes. Secondary metabolites impacting the host might be identified by determining which gene clusters are actively transcribed during infection Possibly some pathways such as the polyketide pathway are blocked with growth on the scalp, leading to reduced sporulation and growth and to formation of pigmented secondary metabolites seen in cultures, which can be restored by repeated transfer on artificial media; sporulation is vitamin B dependent (Zhan *et al* ., 2018).

3.8.4 Variants calling

As a part of the whole genome analysis of (6TR , 7TR) genetic differences (variants) were called between the studied genomes and reference genome for identifying the existence of a single-nucleotide polymorphism (SNPs), Insertions, Deletions and Base changes. In addition, this study identified types of SNPs either transition or transversion variants at the whole genome of studied *Trichophyton mentagrophytes* .

3.8.4.1 Variants calling for *Trichophyton mentagrophytes* (6 TR and 7TR)

According to the general variants count, the results of this study showed the existence of 72055 variants on 6TR genome in comparison with the reference genome. With regard to 7TR genome, 629423 variants were identified compared with reference genome. Interestingly, 7TR genome generated the higher number of variants in contrast to 6TR genome .

According to described data above, the general variants count of studied genomes showed 6TR strain was more relative to the reference genome than 7TR strain , where only 72055 variants were identified. This result explains the huge evolutionary events that occurred on 7TR genome that force this strain to be so far from the reference genome lineage, suggesting that 7TR genome evolved independently from this lineage in spite of they have shared ancestry.

Additionally, the findings of this study revealed that the higher rate of genetic variation for the studied genome was related to SNPs more than Insertions and Deletions.

Furthermore, this result documented the presence of very high rate of SNPs in 7TR genome compared with the reference genome. Additionally, this study noted low rates of insertion-deletion (Indels) in all studied genomes. Generally, the high level of SNPs reflects high point mutation rates (Alwi, 2005). Thus, this study presumes that the most common type of genetic variation in the studied *Trihophyton mentagrophytes* genomes is single-base mutations rather than Indel Mutations.

The level of polymorphism may be due to the mutation rates in dermatophyte species and the species recent emergences from a genotype .

Genetic variability between isolates occur for a varity of reasons such as point mutations ,single nucleotide insertion or deletion .

The effect of genomic changes on translation was variable. The genomic features of SNPs identified, including the SNP position and type, allele length, annotation, coding region and amino acid changes. The number of SNP difference between isolates observed depends on mutation rate and the evolutionary time that has passed. Therefore, the small number of SNP differences observed between our isolates suggests that it is highly likely that these isolates shared a very recent common ancestor.

Single nucleotide polymorphisms (SNPs) is a key method to identify any population, geographical, lineage, drug response or pathogenicity variability among different available genomes. Among different types of SNPs in the genomes, the non-synonymous SNPs, designated as single amino-acid polymorphisms (or SAPs), would result in amino-acid changes and have a direct effect on function of the protein، providing important clues into mechanistic aspects of the disease (Kumar *et al*., 2009) .

Most of the concerns indel events caused by incorporation events, or intervals that are too short to be reliably detected (Zhan *et al*.,2018).

Furthermore, the results of this revealed that SNPs variants covered the higher number of variant counts in all studied genomes (6TR and 7TR), where 70362 (97.65%) of 6TR variants identified as SNPs while only 687 (0.95%) and 1006 (1.4%) of the total number of 6TR variants detected as Insertions and Deletions. Similarly, of a total of 629423 variants determined in 7TR genome, SNPs comprised 565184 (89.79%) while only 32667 (5.19%) Insertions and 31572 (5.02%) Deletions were identified. Number and percentages of variants summarized in table (3-21) and Figure (3-18).

Table (3-21): Comparison of 6TR and 7TR with reference genome to identify variants (Nucleotide Polymorphism, Indels)

Sample	Data	Nucleotide Polymorphism	Indels		Total
		SNP	Insertion	Deletion	
6TR	N	70362	687	1006	72055
	%	97.65%	0.95%	1.40%	100
7TR	N	565184	32667	31572	629423
	%	89.79%	5.19%	5.02%	100

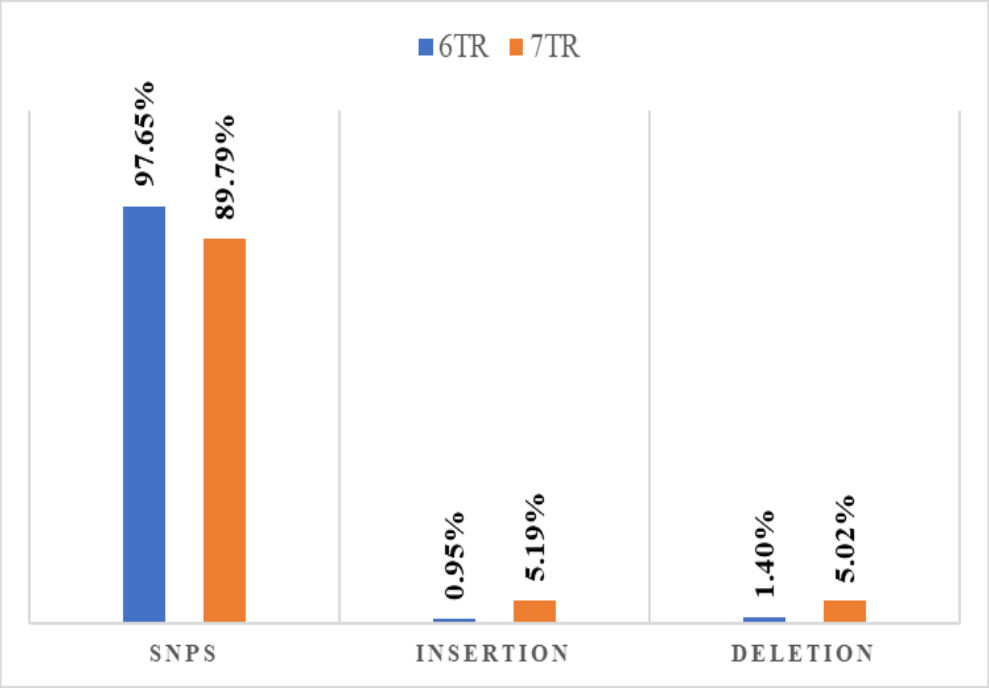


Fig (3-18): percentages Comparison of 6TR and 7TR variants with reference genome

3.8.4.2 Identification of patterns of nucleotide substitution for studied *Trichophyton mentagrophytes* (6TR and 7TR)

After identification of SNPs variants, this study calculated the base change count on every SNPs to identify the type of sequence variation. The results of this study revealed that base substitution among studied *Trichophyton mentagrophytes* genomes (6TR and 7TR) was relative, where majority of base substitution on all the studied genomes revealed the following base changes:

Adenine (A) nucleotide was substituted with Guanine (G) or vice versa, and Thymine (T) was substituted with cytosine (C) or vice versa. In more details, the higher percentages of A substitution in 7TR genomes were A → G substitution with 102850(71.44)%, while the higher base-substitution counts for T nucleotide showed T → C substitution with 103144(71.47). Moreover, in 6TR the higher percentages of A substitution noted as A → C substitution with16243(80.92). similarly, the most common type of T substitution detected as T → G substitution with 16010 (80.61). General Base change results were summarized in Table (3-22) and illustrated in figures (3-19) , (3-20) for each studied genome.

Table(3-22) Base substitution of 6TR and 7TR genomes vs the reference genome

Ref>Alt	6TR	7TR
A>C	16243 (80.92%)	24005 (16.68%)
A>G	941 (4.69%)	102850 (71.44%)
A>T	2890 (14.4%)	17102 (11.88%)
C>A	10832 (73.25%)	21715 (15.87%)
C>G	2990 (20.22%)	15965 (11.69%)
C>T	966 (6.53%)	99110 (72.45%)
G>A	887 (6.14%)	99322 (72.54%)
G>C	2933 (20.22%)	16043 (11.72%)
G>T	10632 (73.57%)	21546 (15.74)
T>A	2936 (14.78%)	17281 (11.97%)
T>C	915 (4.61%)	103144 (71.47%)
T>G	16010 (80.61%)	23902 (16.56%)

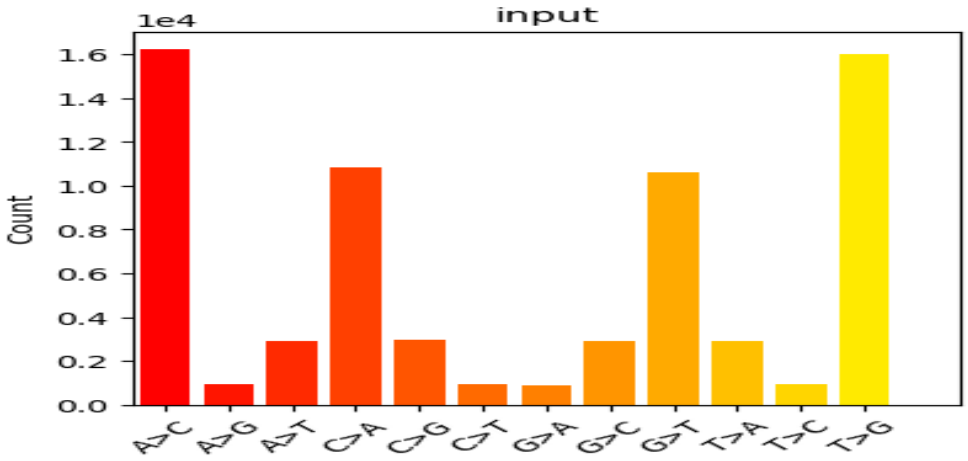


Figure (3-19): Substitution types of 6TR genome vs the reference genome

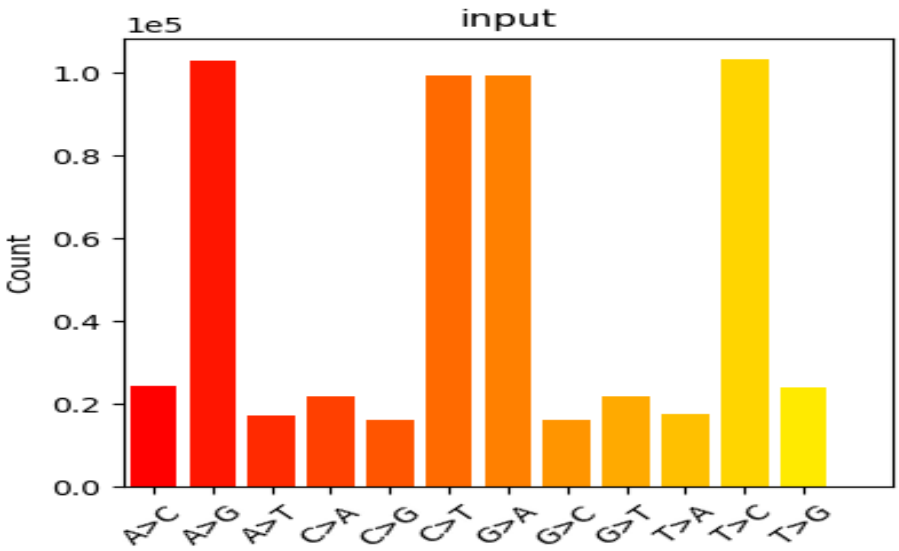


Figure (3-20): Substitution types of 7TR genome vs the reference genome

Additionally, transition and transversion variants were identified to detect patterns of nucleotide substitution. The results of this study revealed that studied genomes showed high rate of Transition substitution compared with Transversion substitution in 7TR while in the 6TR show high rate of transversion substitution compared with transition substitution . With more details in 6TR and 7TR genomes, transition substitution comprised 3709 (5.36%) and 404426

(71.96%),respectively of SNPs variants, while transversion substitution included only 65466 (94.64%) and 157559 (28.04%), respectively of SNPs variants, as shown in Table(3-23) and figure(3-21)

Table(3-23): Distribution of transitions and transversions among the studied genomes (6TR and 7TR)

Genome	Transitions N. (%)	Transversions N. (%)
6TR	3709 (5.36)	65466 (94.64)
7TR	404426 (71.96)	157559 (28.04)

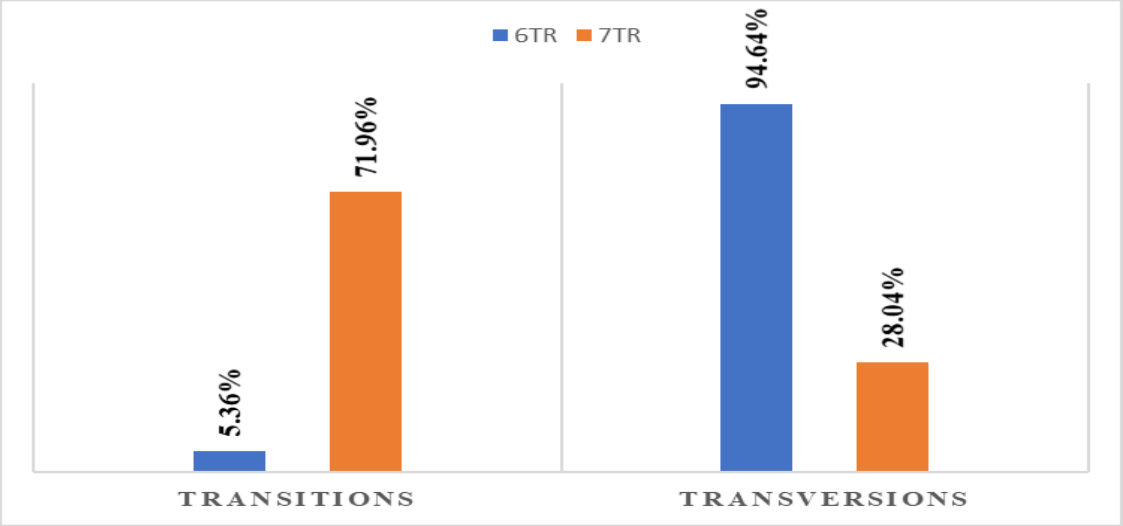


Fig (3-21): Percentages of base substitution type (transitions and transversions) among the studied genomes (6Tr and 7TR)

According above results, there are significant differences among the studied genomes regarding type of sequence variation or patterns of nucleotide substitution, where all studied genomes showed different variation patterns. Moreover, the most common patterns of base substitution in all studied genome were C ↔ T and G ↔ A substitutions in 7TR. While in 6TR the most common patterns of base substitution in studied genome were A↔ C and T ↔ G Based on these results, these patterns represent transition substitution rather than

transversion substitution, where transition variants comprised the higher percentages in comparison with transversion variants in all studied genomes after SNPs analysis.

The high rate of transition substitution could be related to absence repair mechanisms that must revert C ↔ T and G ↔ A transitions.

The total number of SNPs observed among the isolates varies depending on species , in particular for the most homogenetic ones, the sample size, the time span, and the filtering approaches for SNP detection.

Transitions occurred at roughly twice the rate of transversions, similar to results from sequence comparisons; however, several individual transversions are more frequent than the least common. Differences among point mutation rates can also affect the overall G+C content of a genome. For example, when C→T transitions occur at a higher rate than T→C transitions, A,T pairs will accumulate at neutral sites, such as non-coding DNA, and degenerate codon positions. By measuring mutation rates for all possible point mutations, we found a highly bias towards the formation of A,T pairs among transitions, but no bias among transversions . Changes in the patterns of mutations within a species can be exposed by reconstructing the Draft genomes of dermatophytes show very high degrees of conservation, both at nucleotide and at amino acid levels (Martinez *et al*. 2012).

3.8.5 Phylogeny

In the present study, phylogenetic analysis of the studied *Trichophyton mentagrophytes* genomes (6TR and 7TR) was performed to determine the closest representative genomes of *Trichophyton mentagrophytes* TIMM 2789. This strain shared high homology with studied 6TR and 7TR strains , and grouped with the same clade for each one suggesting same clone origin.

Furthermore, the nearest phylogenetic neighbor of *Trichophyton mentagrophytes* to the studied genomes was *Trichophyton mentagrophytes* TIMM 2789 while the furthest neighbor of *Trichophyton* was *Trichophyton tonsurans* CBS 112818 strain. Phylogenetic placement of studied genomes illustrated in figure(3-22).

A closer comparison of the genome sequence of *Trichophyton mentagrophytes* isolates supports a clonal relationship of the population. Phylogenetic analysis of the closely related *Trichophyton mentagrophytes* isolated suggest that the isolates have a similar level of divergence from each other . This supports of the idea that these *Trichophyton mentagrophytes* isolates have likely undergone clonal expansion.

The phylogenetic tree showed that the isolates from this study were tightly clustered together as one group . The *Trichophyton mentagrophytes* isolates from different infection close genetic relationships , and epidemiology or trace back evidence was provided to establish the connection between these *Trichophyton mentagrophytes* isolates.

However, identification of the phylogenetic placement of the studied genomes in Babylon Province of Iraq and determination of the closest representative genomes could facilitate understanding evolution of *Trichophyton mentagrophytes*, that in turn may assist in restoration of control polices and strategies to eliminate *Trichophyton* disease in Iraq.

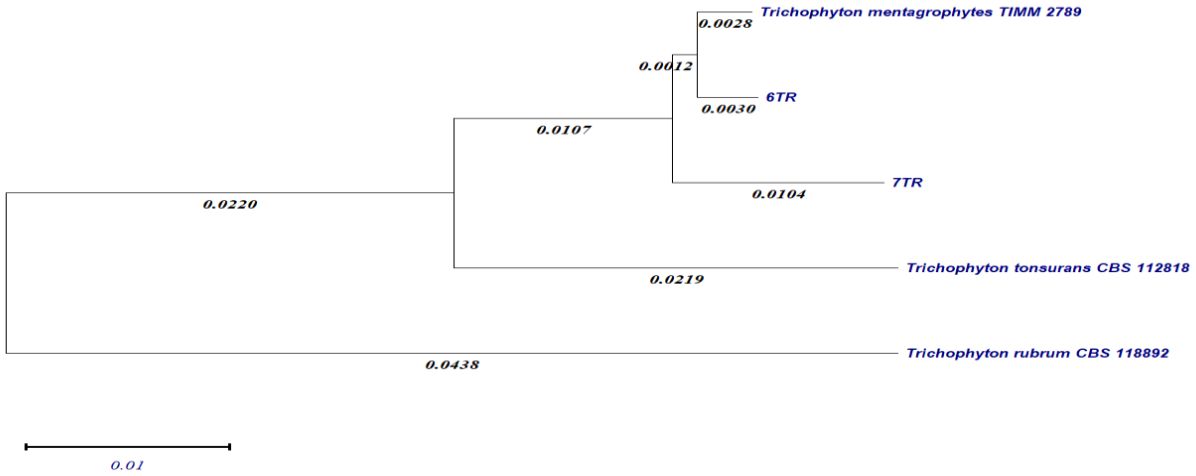


Fig (3-22): The closest reference and representative genomes to 6TR and 7TR genomes to determine the phylogenetic placement of this genome. Black numbers represent red numbers represent branch length. By mega X

Recent gene gain and loss events within the dermatophytes suggest candidates for roles in infection and host specialization, which could help guide the development of new therapies. These species-specific adaptations could be important in host immune system interaction or survival in the environment.

A cautious phylogenetic analysis of sequences is required to resolvethese labelling issues specifically in the *T. mentagrophytes* complex. Expect that genomic and multigene approaches help resolve these issues.Initial approaches using multigene phylogenies by (de Hoog *et al.* 2017) and genome wide analyses (Pchelin *et al*., 2019) seem to be in topological concordance with our phylogenomic observations of the genus members, making the potential of these techniques evident. Even so, increasing the number of public sequences for gene markers and genomes is required for these methods to take a central stage in taxonomic designation. Phylogenetic analysis of the concatenated loci can resolve species boundaries between *T. mentagrophytes* and *T. interdigitale* provided

enough sequences of all the loci from several geographical regions are available in the database (Baert *et al* ., 2019).

Reports of genetic variation contributing to disease susceptibility rather than severity also exist, although full functional validation of these variants is lacking.

Further, phylogeny studies demonstrated trees with a paraphyletic or even polyphyletic *T. mentagrophytes/T. interdigitale* species complex, with *T. tonsurans* and *T. equinum* isolates branching within their clade (Suh *et al* ., 2018) . All four species are being placed in the *T. mentagrophytes* series .However, a recent study using whole genome sequencing (WGS) suggest that *T. mentagrophytes and T. interdigitale* are conspecific (Pchelin *et al* ,.2019).

The phylogenetic clustering is an effective tool that relies on an alignment-free approach for genomic evolution study, the phylogenetic trees acquired are affected by recombinant genes or horizontal gene transfer including plasmid , prophage , and other accessory gene contents (van Vliet and Kusters, 2014) .

The phylogenetic analysis based on WGS-derived SNPs has been shown to provide greater cluster resolution than the gold standard subtyping method, pulsed-field gel electrophoresis (PFGE), resulting in discrimination of outbreak-related human clinical isolates and food or environmental origins .

Conclusions

1- *Trichophyton mentagrophytes* and *Trichophyton rubrum are* most common cutaneous *Trichophyton* infection.

2- The isolates were seen to be able to produce different virulence factors such as Keratinase, Phospholipases , Transcription factor PacC and heat shock protein , this makes the *Trichophyton species* more pathogenic.

3- The healthy condition, age and gender influence the nature and extend of the relationship established with the *Trichophyton*.

4- MLST has emerged as an important tool to study the long-term epidemiology and the population structure and patterns of evolutionary descent. The high genetic variability amongst *Trichophyton species* isolates in this study provides some information on the local dissemination and genetic relatedness.

5- Notwithstanding high discriminatory power, nucleotide changes accumulate in housekeeping genes in long period of time. That is why the allelic profile of isolates persists unchanged over a longer timeframe, which make MLST a desirable tool for global epidemiology.

6- Microbial Genome sequencing is a method that is being used For available genome sequence data allows for the examination of the microevolution of isolates.

7- Whole genome sequence is a helpful tool to assess the phylogenetic relations of *Trichophyton species* , virulence gene evaluation , assist in the molecular epidemiological studies and examination of the microevolution of isolation.

8-The genome sequence of *Trichophyton mentagrophytes* will prove to be evaluable resource for identifying the gene involved various metabolic pathway along with elucidation of molecular mechanism if it

pathogenesis and comparative analysis of *Trichophyton mentagrophytes* strains affecting other parts of the world.

Recommendations

a. Further studies should be done to rapid investigation on *Trichophyton species* directly by PCR without cultivation.

b. Further global investigations covering more isolates and methods like whole genome sequencing would be advisable.

c. Investigation on new antifungal agents to arrest the fungal virulence factors.

d. Detection of putative genes involved in virulence and in resistance to antifungal.

e. Using the laboratory animals for in vivo study is needed to help clarify the role of fungal mutations and genomic rearrangements in *Trichophyton* infection.

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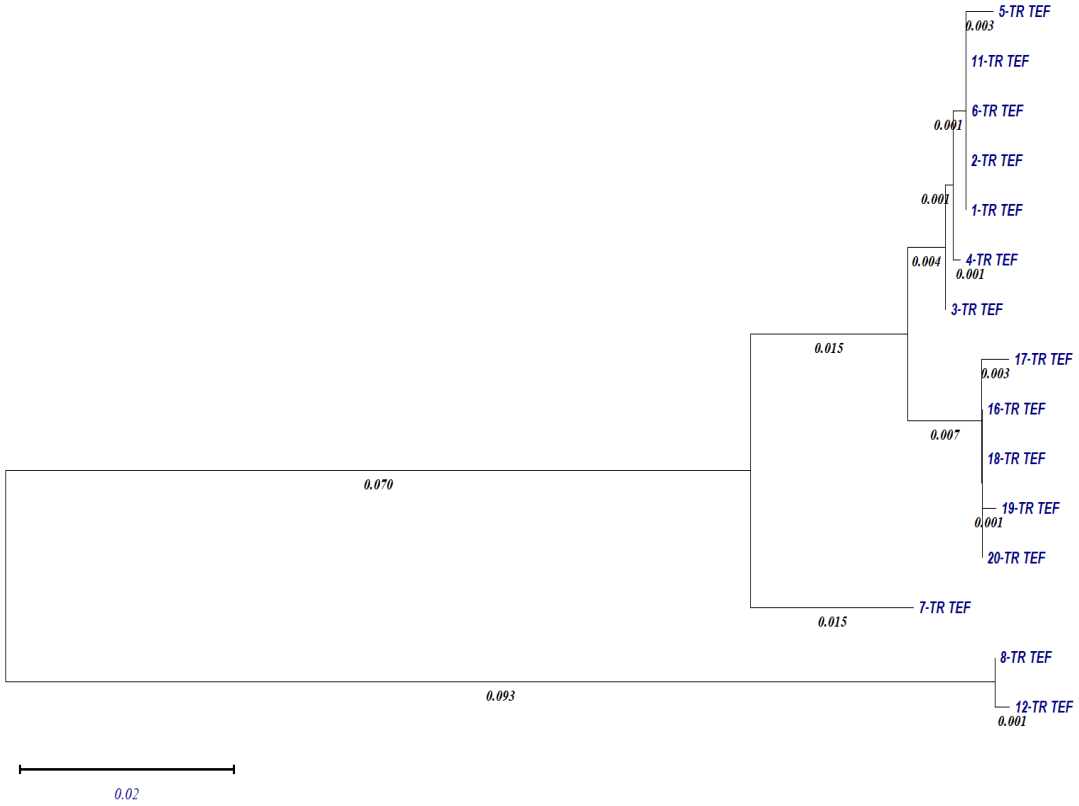
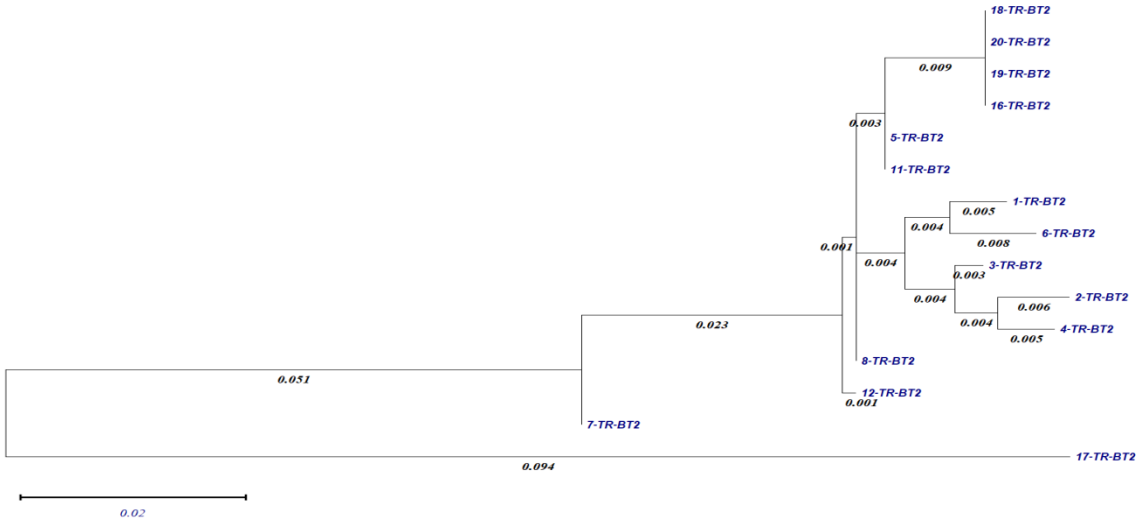
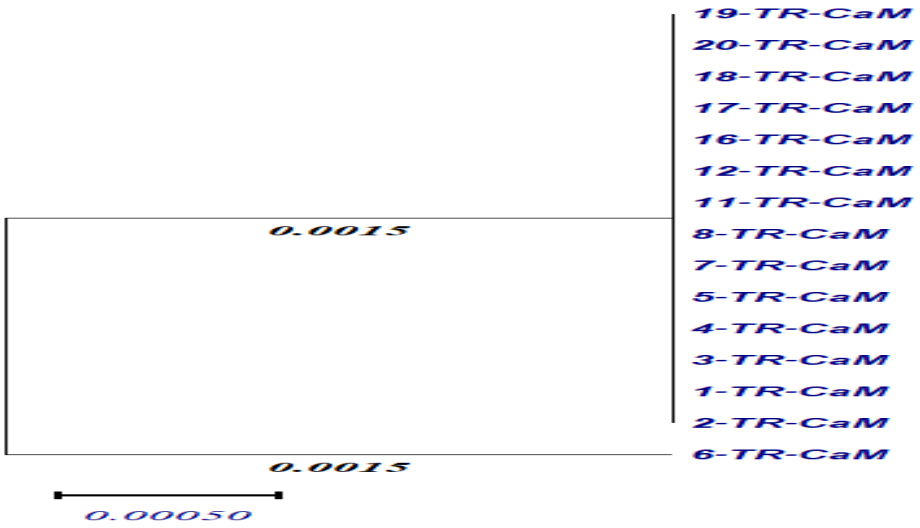
Appendix (1)



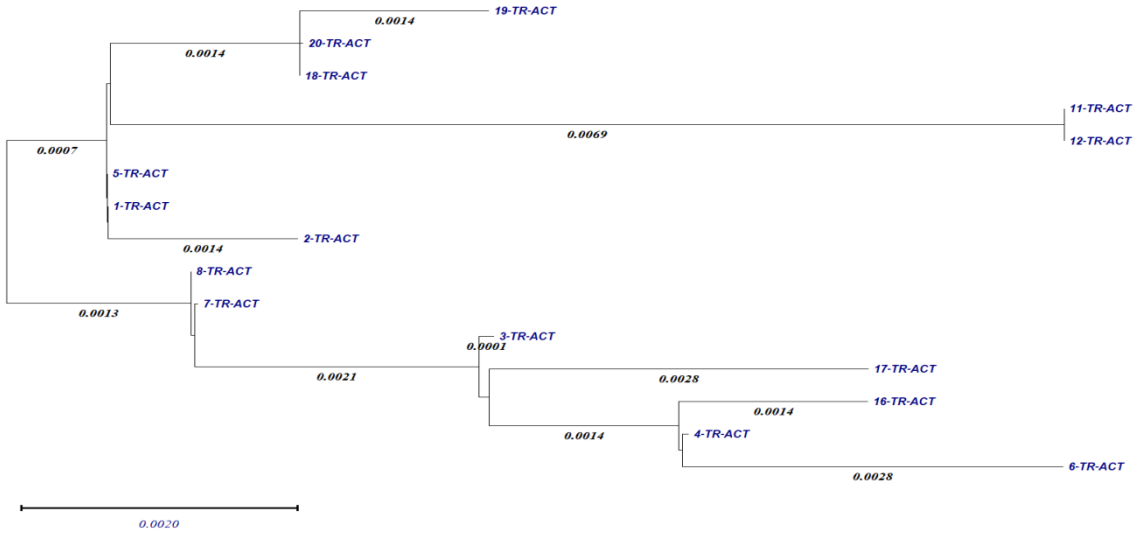
Phylogram analysis based on *TEF-1α* gene sequences from 15 *trichophyton species* isolates by maximum likelihood method



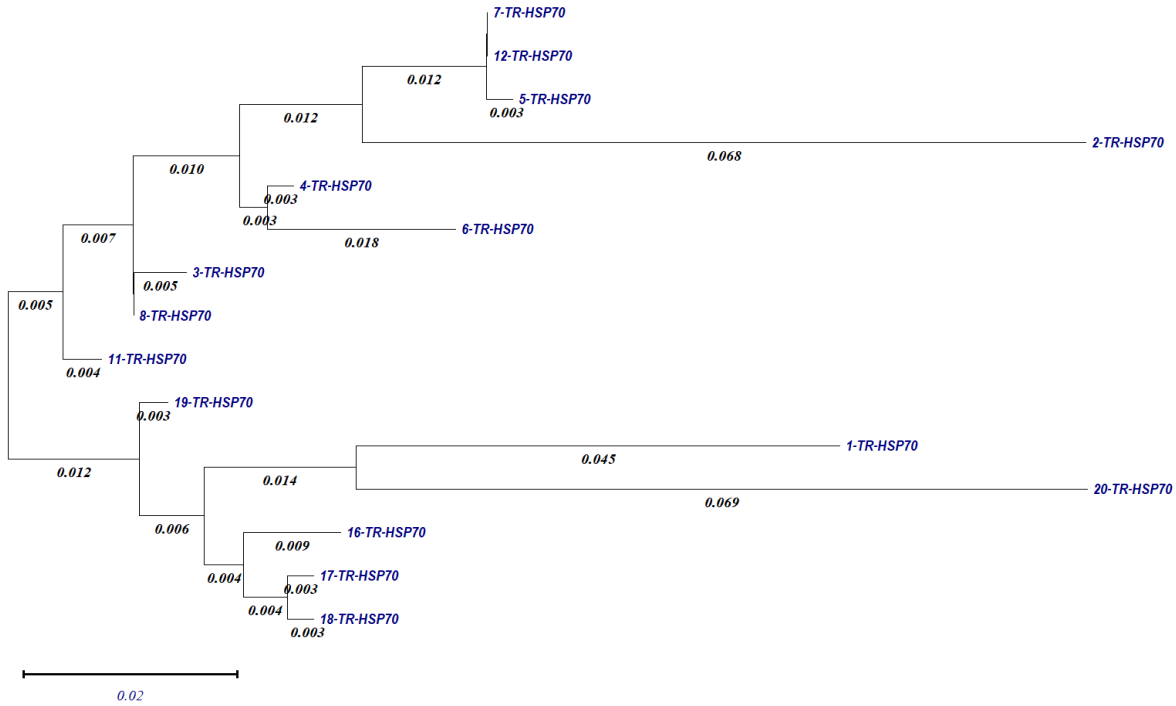
Phylogram analysis based on *Bt2* gene sequences from 15 *trichophyton species* isolates by maximum likelihood method.



Phylogram analysis based on *CaM* gene sequences from 15 *trichophyton species* isolates by maximum likelihood method.

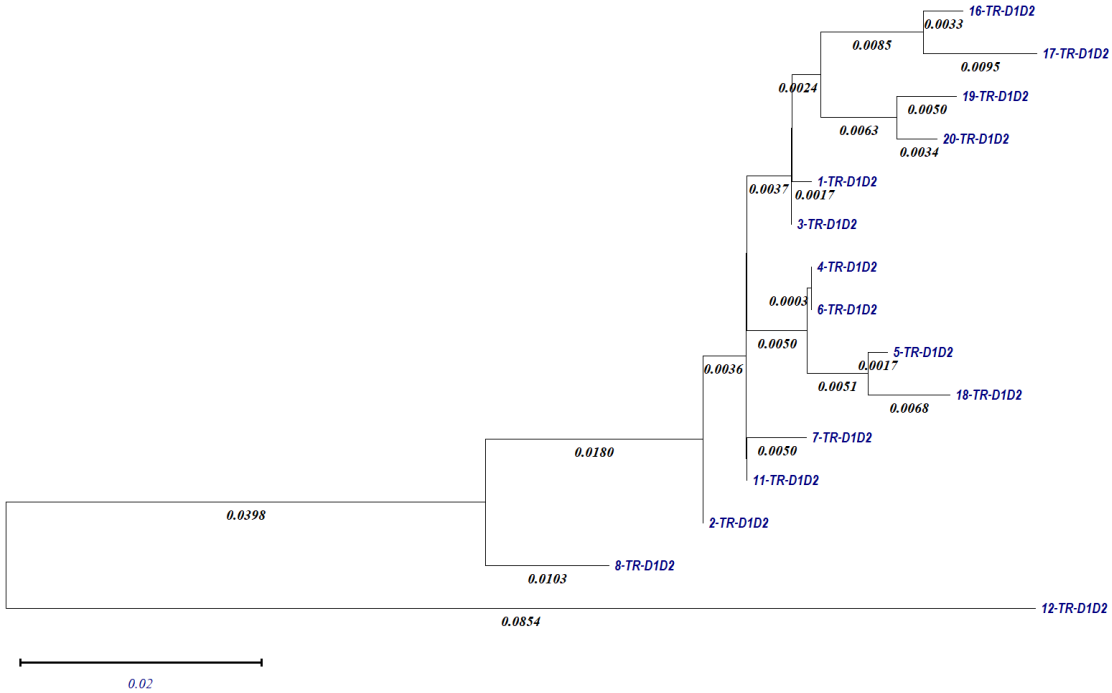


Phylogram analysis based on *ACT* gene sequences from 15 *trichophyton species* isolates by maximum likelihood method.



Phylogram analysis based on *HSP70* gene sequences from 15 *trichophyton species* isolates by maximum likelihood method.

Phylogram analysis based on *ITS* sequences from 15 *trichophyton species* isolates by maximum likelihood method.



Phylogram analysis based on *D1D2* sequences from 15 *trichophyton species* isolates by maximum likelihood method.

Appendix (2)

Alpha-tubulin
TUB1
Aspartic endopeptidase PEP1[...]
PEP1, TRV_03007
Aspartic protease PEP1[...]
PEP1, ARB_05728
Carboxypeptidase 2[...]
MCPB, CARB2
Carboxypeptidase S1 homolog A[...]
SCPA
Carboxypeptidase S1 homolog B[...]
SCPB
cell pattern formation-associated protein stuA
StuA
Chitin synthase[...]
CHS1
CMGC/CDK/CDC2 protein kinase
PABG_01763, PADG_03637
Dipeptidyl peptidase 4[...]
DPP4, MCYG_02383
Dipeptidyl-peptidase 5[...]
DPP5
Dipeptidyl-peptidase V
DPPV
Extracellular metalloprotease ARB_05317[...]
ARB_05317
Extracellular metalloprotease ARB_07495[...]
ARB_07495
Extracellular metalloprotease MGYG_07913[...]
MGYG_07913
Extracellular metalloprotease TRV_06892[...]
TRV_06892
Extracellular metalloprotease TRV_07111[...]
TRV_07111
Extracellular metalloproteinase 1[...]
MEP1, MCYG_07415
Extracellular metalloproteinase 2[...]
MEP2
Extracellular metalloproteinase 3[...]
MEP3, MCYG_04620
Extracellular metalloproteinase 4[...]
MEP4
Extracellular metalloproteinase 5[...]
MEP5
G protein alpha subunit

Fga1
G-alpha subunit
Gap1
glutamine--fructose-6-phosphate transaminase (isomerizing)[...]
AFUA_6G06340, HCAG_04088, PABG_01388
GTP-binding nuclear protein
PABG_04417
PADG_04810
GTP-binding protein ypt1
BDCG_07079
HCAG_06941
PABG_07851
PADG_08342
Guanine nucleotide-binding protein alpha subunit[...]
fga1, BFJ68_g109, BFJ69_g9200, BFJ70_g13830, BFJ71_g8629, FOXYS1_2808, FRV6_12363
Leucine aminopeptidase 1[...]
LAP1
Leucine aminopeptidase 2[...]
LAP2
Mannose-1-phosphate guanyltransferase[...]
BDCG_08925
HCAG_01552
PABG_03197
PADG_01745
Metallocarboxypeptidase A[...]
MCPA
MCPA, MCYG_06789
Metallocarboxypeptidase A-like protein ARB_03789[...]
ARB_03789
Metallocarboxypeptidase A-like protein MCYG_01475[...]
MCYG_01475
Metallocarboxypeptidase A-like protein TRV_02598[...]
TRV_02598
Mitogen-activated protein kinase PMK1[...]
PMK1
mitogen-activated protein kinase[...]
Amk1
BMP1
CHK1
CMK1
fmk1
mk1
ptk1
vmk1
Neutral protease 2 homolog MGYG_03465[...]
MGYG_03465

Probable aspartic-type endopeptidase ARB_07403[...]
ARB_07403
Probable aspartic-type endopeptidase CTSD[...]
CTSD, ARB_01619
CTSD, TRV_03534
Probable aspartic-type endopeptidase OPSB[...]
OPSB, ARB_04170
OPSB, TRV_06035
Probable aspartic-type endopeptidase TRV_06366[...]
TRV_06366
Probable carboxypeptidase 2[...]
MCPB, ARB_02407
MCPB, TRV_02159
Probable dipeptidyl peptidase 4[...]
DPP4, ARB_06110
DPP4, TRV_00096
Probable dipeptidyl-peptidase 5[...]
DPP5, ARB_06651
DPP5, TRV_02418
Probable extracellular metalloproteinase 1[...]
MEP1, ARB_02406
MEP1, TRV_02160
Probable extracellular metalloproteinase 2[...]
MEP2, ARB_01382
MEP2, TRV_01237
Probable extracellular metalloproteinase 3[...]
MEP3, ARB_05085
MEP3, TRV_06691
Probable extracellular metalloproteinase 4[...]
MEP4, ARB_00762
MEP4, TRV_00081
Probable extracellular metalloproteinase 5[...]
MEP5, ARB_06472
MEP5, TRV_07092
Probable leucine aminopeptidase 1[...]
LAP1
LAP1, ARB_03568
LAP1, TRV_06599
Probable leucine aminopeptidase 2[...]
LAP2, ARB_00494
LAP2, TRV_01590
Probable leucine aminopeptidase ARB_00576[...]
ARB_00576
Probable leucine aminopeptidase ARB_01443[...]
ARB_01443
Probable leucine aminopeptidase ARB_03492[...]
ARB_03492

Probable leucine aminopeptidase TRV_02148.1[...]
TRV_02148.1
Probable leucine aminopeptidase TRV_05286[...]
TRV_05286
Probable leucine aminopeptidase TRV_05750[...]
TRV_05750
Probable metallocarboxypeptidase A[...]
MCPA, ARB_07026/ARB_07027
MCPA, TRV_07931
Probable neutral protease 2 homolog A[...]
NpII-A
Probable neutral protease 2 homolog ARB_00849[...]
ARB_00849
Probable neutral protease 2 homolog ARB_03949[...]
ARB_03949
Probable neutral protease 2 homolog ARB_04769[...]
ARB_04769
Probable neutral protease 2 homolog ARB_05817[...]
ARB_05817
Probable neutral protease 2 homolog B[...]
NpII-B
Probable neutral protease 2 homolog TRV_02539[...]
TRV_02539
Probable neutral protease 2 homolog TRV_05367[...]
TRV_05367
Probable neutral protease 2 homolog TRV_06370[...]
TRV_06370
Probable tripeptidyl-peptidase SED2[...]
SED2, ARB_05765
SED2, TRV_04476
Probable tripeptidyl-peptidase SED3[...]
SED3, ARB_04677/ARB_04678
SED3, TRV_03120
Probable tripeptidyl-peptidase SED4[...]
SED4, ARB_04101
SED4, TRV_03885
Probable vacuolar protease A[...]
PEP2, ARB_02919
PEP2, TRV_05606
Rho-GTPase
Rac
ribose-phosphate diphosphokinase[...]
BDCG_08199
RNA polymerase-associated protein LEO1
ARB_08005
squalene epoxidase SE
SE

Squalene monooxygenase[...]
(blank)
Subtilisin-like protease 1[...]
SUB1
SUB1, MGYG_02140
Subtilisin-like protease 11[...]
SUB11, ARB_06111
SUB11, TRV_00097
Subtilisin-like protease 12[...]
SUB12, ARB_06416
SUB12, TRV_01047
Subtilisin-like protease 2[...]
SUB2
SUB2, ARB_01495
SUB2, MCYG_08690
SUB2, MGYG_06576
SUB2, TRV_08059/TRV_05599
Subtilisin-like protease 3[...]
SUB3
SUB3, ARB_00701
SUB3, MCYG_04040
SUB3, MGYG_02570
SUB3, TRV_07976
Subtilisin-like protease 4[...]
SUB4
SUB4, ARB_01032
SUB4, MCYG_07213
SUB4, MGYG_04715
SUB4, TRV_02781
Subtilisin-like protease 5[...]
SUB5
SUB5, ARB_02223
SUB5, MCYG_08753
SUB5, MGYG_07290
SUB5, TRV_00550
Subtilisin-like protease 6[...]
SUB6
SUB6, ALP1
SUB6, tri m 2
SUB6, TRV_02343
Subtilisin-like protease 7[...]
SUB7
SUB7, ALP2
SUB7, ARB_06076
SUB7, TRV_00296
Subtilisin-like protease 8[...]
SUB8, ARB_00777

SUB8, MCYG_02070
SUB8, TRV_07778
subtilisin-like protease SUB6
SUB6
T-complex protein 1 subunit theta
HCAG_01355
Transcriptional activator hacA
HacA
Tripeptidyl-peptidase SED2[...]
SED2, MCYG_00184
Tubulin alpha chain
BDCG_08660
Tubulin beta chain
TUB1
tub2
Ubiquitin
UBI4, CAALFM_C307270CA, orf19.14063
zinc-responsive activating factor ZafA
ZafA

Appendix (3)

Alpha-tubulin
TUB1
Aspartic endopeptidase PEP1[...]
PEP1, TRV_03007
Aspartic protease PEP1[...]
PEP1, ARB_05728
Carboxypeptidase 2[...]
MCPB, CARB2
Carboxypeptidase S1 homolog A[...]
SCPA
SCPA, ARB_04046
SCPA, TRV_05031
Carboxypeptidase S1 homolog B[...]
SCPB
SCPB, ARB_06019
SCPB, MCYG_02825
cell pattern formation-associated protein stuA
StuA
Chitin synthase[...]
CHS1
CMGC/CDK/CDC2 protein kinase
PABG_01763
PADG_03637
Dipeptidyl peptidase 4[...]

DPP4
DPP4, MCYG_02383
Dipeptidyl-peptidase 5[...]
DPP5
DPP5, MCYG_01626
DPPV
Dipeptidyl-peptidase V
DPPV
Extracellular metalloprotease ARB_05317[...]
ARB_05317
Extracellular metalloprotease MGYG_07913[...]
MGYG_07913
Extracellular metalloprotease TRV_06892[...]
TRV_06892
Extracellular metalloproteinase 1[...]
MEP1
Extracellular metalloproteinase 2[...]
MEP2
Extracellular metalloproteinase 3[...]
MEP3
MEP3, MCYG_04620
Extracellular metalloproteinase 4[...]
MEP4
Extracellular metalloproteinase 5[...]
MEP5
G protein alpha subunit
(blank)
glutamine--fructose-6-phosphate transaminase (isomerizing)[...]
AFUA_6G06340
HCAG_04088
PABG_01388
PADG_03984
GTP-binding nuclear protein
PABG_04417
PADG_04810
GTP-binding protein ypt1
BDCG_07079
HCAG_06941
PABG_07851
PADG_08342
Guanine nucleotide-binding protein alpha subunit[...]
fga1, BFJ68_g109, BFJ69_g9200, BFJ70_g13830, BFJ71_g8629, FOXYS1_2808, FRV6_12363
Leucine aminopeptidase 1[...]
LAP1
Leucine aminopeptidase 2[...]
LAP2

Mannose-1-phosphate guanyltransferase[...]
BDCG_08925
HCAG_01552
PABG_03197
PADG_01745
Metallocarboxypeptidase A[...]
MCPA
MCPA, MCYG_06789
Metallocarboxypeptidase A-like protein ARB_03789[...]
ARB_03789
Metallocarboxypeptidase A-like protein MCYG_01475[...]
MCYG_01475
Metallocarboxypeptidase A-like protein TRV_02598[...]
TRV_02598
Mitogen-activated protein kinase PMK1[...]
PMK1
Mitogen-activated protein kinase[...]
Amk1
BMP1
CHK1
CMK1
fmk1
mk1
ptk1
vmk1
Neutral protease 2 homolog MGYG_03465[...]
MGYG_03465
Probable aspartic-type endopeptidase ARB_07403[...]
ARB_07403
Probable aspartic-type endopeptidase CTSD[...]
CTSD, ARB_01619
CTSD, TRV_03534
Probable aspartic-type endopeptidase OPSB[...]
OPSB, ARB_04170
OPSB, TRV_06035
Probable aspartic-type endopeptidase TRV_06366[...]
TRV_06366
Probable carboxypeptidase 2[...]
MCPB, ARB_02407
MCPB, TRV_02159
Probable dipeptidyl peptidase 4[...]
DPP4, ARB_06110
DPP4, TRV_00096
Probable dipeptidyl-peptidase 5[...]
DPP5, ARB_06651
DPP5, TRV_02418
Probable extracellular metalloproteinase 1[...]

MEP1, ARB_02406
MEP1, TRV_02160
Probable extracellular metalloproteinase 2[...]
MEP2, ARB_01382
MEP2, TRV_01237
Probable extracellular metalloproteinase 3[...]
MEP3, ARB_05085
MEP3, TRV_06691
Probable extracellular metalloproteinase 4[...]
MEP4, ARB_00762
MEP4, TRV_00081
Probable extracellular metalloproteinase 5[...]
MEP5, ARB_06472
MEP5, TRV_07092
Probable leucine aminopeptidase 1[...]
LAP1
LAP1, ARB_03568
LAP1, TRV_06599
Probable leucine aminopeptidase 2[...]
LAP2, ARB_00494
LAP2, TRV_01590
Probable leucine aminopeptidase ARB_00576[...]
ARB_00576
Probable leucine aminopeptidase ARB_01443[...]
ARB_01443
Probable leucine aminopeptidase ARB_03492[...]
ARB_03492
Probable leucine aminopeptidase TRV_02148.1[...]
TRV_02148.1
Probable leucine aminopeptidase TRV_05286[...]
TRV_05286
Probable leucine aminopeptidase TRV_05750[...]
TRV_05750
Probable metallocarboxypeptidase A[...]
MCPA, ARB_07026/ARB_07027
MCPA, TRV_07931
Probable neutral protease 2 homolog A[...]
NpII-A
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ARB_00849
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ARB_03949
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ARB_04769
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ARB_05817
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NpII-B
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TRV_02539
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SUB2, TRV_08059/TRV_05599
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SUB3, ARB_00701

SUB3, MCYG_04040
SUB3, MGYG_02570
SUB3, TRV_07976
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SUB5, MGYG_07290
SUB5, TRV_00550
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SUB6, tri m 2
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SUB7, ALP2
SUB7, ARB_06076
SUB7, TRV_00296
Subtilisin-like protease 8[...]
SUB8, ARB_00777
SUB8, MCYG_02070
SUB8, TRV_07778
Subtilisin-like protease 9[...]
SUB9, ARB_03790
SUB9, MGYG_04593
SUB9, TRV_02597
subtilisin-like protease SUB6
SUB6
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