Polymorphism analysis of virulence-related genes among *Candida tropicalis* isolates

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Abstract

Background: Adhesion, biofilm formation, yeast-hyphal transition, secretion of enzymes, and hemolytic activity are all considered important factors in *Candida tropicalis* infection. However, DNA sequence data for this pathogen are limited. In this study, the polymorphism and heterogeneity of genes agglutinin-like sequences *(ALS)2*, Lipase *(LIP)1*, *LIP4*, and secretory aspartyl proteinase tropicalis *(SAPT)1-4* as well as the relationship between phenotype and genotype were analyzed.

Methods: This study started in August 2013, and ended in July 2017. The complete length of *ALS2*, *LIP1*, *LIP4*, and *SAPT1-4* of 68 clinical *C. tropicalis* isolates was sequenced. Single nucleotide polymorphisms (SNPs) as well as insertions and deletions (indels) were identified within these genes. In addition, phenotypic characteristics of the virulent factors, including adhesion and the secretion of aspartyl proteinases and phospholipases, were determined.

Results: There were 73, 24, 17, 16, 13, and 180 SNPs in the genes *LIP1*, *LIP4*, *SAPT1*, *SAPT2*, *SAPT3*, and *SAPT4*, respectively. Furthermore, 209 SNPs were identified in total for the gene *ALS2*. Interestingly, large fragment deletions and insertions were also found in *ALS2*. Isolate FXCT 01 obtained from blood had deletions on all 4 sites and showed the lowest adhesion ability on the polymethylpentene surface. In addition, isolates with deletions in the regions 1697 to 1925 and 2073 to 2272 bp displayed relatively low abilities for adhesion and biofilm formation, and this phenotype correlated with the deletions found in *ALS2*. *LIP1*, *SAPT4*, and *ALS2* displayed great heterogeneity among the isolates. Large deletions found in gene *ALS2* appeared to be associated with the low ability of adhesion and biofilm formation of *C. tropicalis*.

Conclusion: This study might be useful for deeper explorations of gene function and studying the virulent mechanisms of *C. tropicalis*.

Keywords: *Candida tropicalis*; Virulence-related genes; Phylogenetic analysis; Gene *ALS*; Gene *LIP*; Gene *SAP*

Introduction

Candida species are considered to be the 4th most commonly isolated organisms from blood-stream infections in the United States, and the 6th most common in Europe; it is responsible for the overwhelming majority of urinary tract infections.[1] However, infections caused by non-*Candida albicans Candida* (NCAC) species, including *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. dubliniensis*, and *C. parapsilosis*, are increasing. *Candida tropicalis* accounts for 40% to 70% of mortality caused by blood infections, which is associated with many other factors, such as leukemia, neutropenia, central venous catheters, parenteral nutrition, and extended time in intensive care units.[2]*Candida tropicalis* has emerged as the second or third most common agent of candidemia, mainly in oncology patients, and is often associated with nosocomial urinary-tract infections.[3]*Candida tropicalis* is close to *C. albicans* genetically, and it is able to form germ tubes, pseudohyphae, and hyphae.[4] Adhesion to host surfaces (epithelial cells and medical devices), biofilm formation, the ability to undergo a morphological switch between yeast and hyphal growth, secretion of enzymes (proteases and phospholipases), and hemolytic activity are all considered important factors in *C. tropicalis* infection.[5]*ALS1-3*, *LIP1-10*, and *SAPT1-4* encode adhesins, lipases, and secreted aspartyl proteinases (Sap) of *C. tropicalis*, respectively.[6] However, limited DNA sequence data have been published, and no study has investigated the role of these genes in the virulence of *C. tropicalis*.

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Until recently, little was known about the role of these genes involved in the virulence of *C. tropicalis*. Many studies have been performed for *SAPT1-4* genes, including DNA sequencing and protein function studies.[7] However, limited DNA sequence data for *ALS1-3* and *LIP* genes of *C. tropicalis* are available in GenBank. Moreover, there are only 2 whole genomes available for *C. tropicalis*. In this study, we designed primers for and amplified the genes *SAPT1-4*, *ALS1-3*, *LIP1*, and *LIP4* based on the whole-genome sequences of *C. tropicalis* available.[4] We intend to study the heterogeneity of each virulence-related gene family and explore the relationship between the genotypes and phenotypes of *C. tropicalis*.

Methods

Ethical approval

The study (from August 2013 to July 2017) was conducted in accordance with the *Declaration of Helsinki* and was approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. As retrospective study and data analysis were performed anonymously, this study was exempt from the requirement of informed consent from patients.

C. tropicalis isolates used in this study

A total of 68 *C. tropicalis* isolates were obtained from 3 different general hospitals during the period of August 2013–July 2014 in Beijing, China. These strains belong to the archive collection of the Chinese Centre for Control and Prevention. The origins of these strains were diverse, including 31 from sputum, 10 from urea, 7 from feces, 4 from vaginal secretion, 3 from blood, 3 from drainage, 2 from throat swab, 1 from prostatic secretion, 1 from sanies, and 6 from other unknown. All isolates were identified by internal transcribed spacer (ITS) sequencing and AUX 20C (BioMérieux, Lyon, SA, France) in our lab. The universal primers *ITS1* and *ITS4* were used to amplify and sequence the ITS fragment in both directions.[8] The strains were stored at 280°C in brain-heart infusion (Oxoid, Basingstoke, UK). The isolates were maintained on Sabouraud agar (Oxoid) during the study. *In vitro* enzymatic activities (aspartyl protease, phospholipase, and hemolytic activities), adhesion, and biofilm formation were analyzed for all *C. tropicalis* isolates.

Primer design, amplification of target genes, and sequence assembly

Primers were designed to amplify the genes *ALS2*, *SAPT1*, *SAPT4*, *LIP1*, and *LIP4* based on the related DNA sequences and whole genomes obtained from GenBank. Primers reported previously were used to amplify genes *SAPT2* and *SAPT3*.[7] The total genomic DNA of the isolates was extracted using a Yeast DNA purification kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The concentration of the genomic DNA samples was estimated using a spectrophotometer by reading the absorbance at 260 nm. DNA extracts were stored at −20°C until use. *SAPT1-3* and *ALS2* were amplified through regular polymerase chain reaction (PCR), while *SAPT4*, *LIP1*, and *LIP4* were amplified through long and accurate PCR (LA-PCR). Amplification was carried out in a final reaction volume of 50 μl that consisted of 25 μl of Premix rTaq or Premix LA Taq (TAKARA, Tokyo, Japan), 20 μl of dH_2O, 3 μl of template DNA, and 1 μl of the forward and reverse primers each. Amplification conditions are shown in Table 1. The amplified fragments were purified using a PCR purification kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions. Both strands of the purified fragments were sequenced using the same primers as those used in the initial amplification. DNA sequencing was performed with an ABI 3730 DNA analyzer (Applied Biosystems, Beverly, MA, USA).

For some isolates, it was difficult to get high-quality sequences using PCR products for direct sequencing; for these isolates, the genes were 1st cloned to obtain the whole-gene sequences. Here, pGEM®-T Easy vector systems II (Promega, Fitchburg, WI) was applied as the cloning vector. The details of constructing the cloned vector and selecting positive clones to be sequenced were in accordance with the methods described earlier.

To get the whole length of each gene, primer walking strategy was applied for all genes except *ALS2*. Based on the obtained sequences from the 1st primers, the 2nd pair of primers was designed, and then PCR products were sent for sequencing. Then, the following pair of primers was designed based on the newly acquired sequences until the full length of the gene was obtained. Five pairs of primers were designed to amplify the whole length of gene *ALS2* [Table 1]. Software DNA Star (http://www.dnastar.com) was used to assemble DNA sequences. Primers that were designed are shown in Table 1.

Analysis of virulence phenotype and the corresponding genes

The phenotypic characteristics of virulence factors, including adhesion and the secretion of aspartyl proteinases and phospholipases, were determined as described previously.[9] In brief, the hydrolytic activity was determined on plates containing specific substrates by observing precipitation or the formation of a translucent halo. Adhesion was analyzed on both abiotic (polystyrene) and biotic (human urinary bladder epithelial cell) surfaces.[10] The complete lengths of the genes *SAPT1-4*, *LIP1*, *LIP4*, and *ALS2* from all 68 *C. tropicalis* isolates were aligned and further analyzed using MEGA 6 separately. Single nucleotide polymorphisms (SNPs) in *SAPT1-4*, *LIP1*, and *LIP4* were also determined using MEGA 6, and the results were displayed by Origin Viewer software (OriginLab Corporation, Northampton, MA, USA).

Phylogenetic analysis of *C. tropicalis* isolates based on their virulence-related genes

The genetic relationships among the 68 strains were determined using the neighbor-joining (NJ) method in MEGA 6 software based on each virulence gene family

Table 1: Primers used in this study.

Genes	Sequence 5′-3′	Length of products (bp)	PCR conditions
SAPT1	F: TGTTGTTATTGTAGATGGAGGAC	1734	94°C, 4 min; 94°C, 1 min; 53°C, 45 s; 72°C, 100 s; 35 cycles 72°C, 10 min; 4°C, ∞
	R: GTTGGTCCATTTATTGGTTCAT		
SAPT2[*]	F: TCCGCTATTTATTCACCAGA	1530	
	R: TGAGGGCTAGTACCAATCGT		
SAPT3[*]	F: AATTGGAATATAAATACGCT	1325	
	R: GGGGGTGAAACTACAATTTA		
SAPT4	F: GTCAAATGTCGCTGCTGGAGGAA	1970	Same with *LIP1,4*
	R: ATCGTGGTTGGAGATTTGTATGA		
ALS2[†]	F1:GGTAAAAGAAGTCGGATACGCAT	1214	94°C, 4 min; 94°C, 1 min, 53°C, 45 s; 72°C, 2 min, 35 cycles 72°C, 10 min; 4°C, ∞
	R1: CTACATCCGTTGCCATTACTATT		
	F2: AATGAATAAGTGTAGTATGTCCA	1516	
	R2: TCTTTTTGAACCTACAACATACA		
	F3:AGCAGTAGTGTAAAAGTCAGTCA	1426	Annealing temp. 56°C, same conditions with 1,2
	R3: ATTATTCCACCATTACGGCAAC		
	F4: CATCTTCAAATAATGTCGTCGTA	936	Same with primer 1,2
	R4: TTTGGTGTTGGTGGTTCAGGAT		
	F5: AAAAAATGGGCGATACCCTTCTG	1345	Same with primer 3
	R5: TAATGGTCAGGCGGGCGTGTCT		
LIP1	F: CCAAGGAGTCTATGGCTCAGTTA	1945	94°C, 1 min; 98°C, 10 s; 50°C, 10 min; 35 cycles 72°C, 10 min; 4°C, ∞
	R: TAAGTGTAAAGTTGTCGGTGTTC		
LIP4	F: AAACCAGCGACACCAACCTACAA	2277	
	R:TGGTGGAAAGACAGGTCGCAGTT		

[*] Primers reported in reference. [†] Five primer pairs were designed to cover the whole length of the gene. *ALS*: Agglutinin-like sequences; *LIP*: Lipase; *SAPT*: Secretory aspartyl proteinase tropicalis.

independently. In addition, data of every single gene from each virulence gene family of *C. tropicalis* were conjoined into a single sequence (ie, the sequences of *LIP1* and *LIP4* were combined into LIP, the sequences of *SAPT1-4* were combined into SAP), and then each base in the sequence was rewritten twice for a homozygous (A, C, G, or T) datum or as the 2 component bases for a heterozygous (K, M, R, S, W, Y) datum. These revised sequences were then used to generate the genetic distance matrices.

Analysis

The DNA sequences of all tested genes were assembled using Software DNA Star (http://www.dnastar.com). And then they were further aligned by MEGA 6. In addition, SNPs of these genes were also determined by MEGA 6 and results were displayed by Origin Viewer software. The genetic relationship among the 68 strains was determined using NJ tree in MEGA 6 software based on each virulent gene family.

Results

We successfully amplified the whole length of each gene from all 68 *C. tropicalis* isolates. Primers used in this study are displayed in Table 1. The length of *ALS2* in the *C. tropicalis* isolates was 4071 bp, *LIP1* was 1398 bp, *LIP4* was 1392 bp, *SAPT1* was 1185 bp, *SAPT2* was 1820 bp, *SAPT3* was 1200 bp, and *SAPT4* was 1920 bp. All 68 *C. tropicalis* isolates were divided into 60, 66, 36, 21, 30, 35, and 64 genotypes according to *ALS2*, *LIP1*, *LIP4*, *SAPT1*, *SAPT2*, *SAPT3*, and *SAPT4*, respectively. The A+T content (60–65%) was much higher than the G+C content (35–40%) in all these genes. All these DNA sequences have been submitted to GenBank and their accession numbers are shown in Table 2.

Furthermore, point mutations and SNPs were discovered in all gene sequences. There were 73, 24, 17, 16, 13, and 180 SNPs found in *LIP1*, *LIP4*, *SAPT1*, *SAPT2*, *SAPT3*, and *SAPT4*, respectively. The locations of the SNPs within each gene and the number of isolates with each SNP site are displayed in Figure 1. In the *SAPT* gene family, there were several suspected SNPs that were identical among the 68 tested isolates but were distinct from the reference strain; there were 11 SNPs in *SAPT1*, 1 in *SAPT2*, 2 in *SAPT3*, and 2 in *SAPT4* [Figure 1]. Furthermore, 209 SNPs in total were identified in *ALS2*. Interestingly, *ALS2* had large fragment deletions and insertions [Figure 2]. There were 2 fragments of insertion located in the 1731 to 1841 and 2163 to 2273 bp regions. Isolate CYCT02 had insertion on both sites, while ZRCT45, 03, 06, 07, and 09 only had 1 insertion on either of the 2 sites [Figure 2]. Four fragments of deletion with lengths of 107, 228, 110, and 199 bp were

Table 2: GenBank accession numbers of each gene from 68 *Candida tropicalis* isolates.

No. of strains	SAPT1	SAPT2	SAPT3	SAPT4	LIP1	LIP4	ALS2	DST
ZRCT01	MF924934	MF925002	MF925070	MF925142	MF924802	MF924870	MF924730	279
ZRCT02	MF924935	MF925003	MF925071	MF925143	MF924803	MF924871	MF924731	291
ZRCT03	MF924936	MF925004	MF925072	MF925144	MF924804	MF924872	MF924732	314
ZRCT04	MF924937	MF925005	MF925073	MF925145	MF924805	MF924873	MF924733	316
ZRCT05	MF924938	MF925006	MF925074	MF925146	MF924806	MF924874	MF924734	271
ZRCT06	MF924939	MF925007	MF925075	MF925147	MF924807	MF924875	MF924735	409
ZRCT07	MF924940	MF925008	MF925076	MF925148	MF924808	MF924876	MF924736	281
ZRCT08	MF924941	MF925009	MF925077	MF925149	MF924809	MF924877	MF924737	286
ZRCT09	MF924942	MF925010	MF925078	MF925150	MF924810	MF924878	MF924738	410
ZRCT10	MF924943	MF925011	MF925079	MF925151	MF924811	MF924879	MF924739	319
ZRCT11	MF924944	MF925012	MF925080	MF925152	MF924812	MF924880	MF924740	295
ZRCT12	MF924945	MF925013	MF925081	MF925153	MF924813	MF924881	MF924741	272
ZRCT13	MF924946	MF925014	MF925082	MF925154	MF924814	MF924882	MF924742	312
ZRCT14	MF924947	MF925015	MF925083	MF925155	MF924815	MF924883	MF924743	318
ZRCT15	MF924948	MF925016	MF925084	MF925156	MF924816	MF924884	MF924744	298
ZRCT16	MF924949	MF925017	MF925085	MF925157	MF924817	MF924885	MF924745	315
ZRCT17	MF924950	MF925018	MF925086	MF925158	MF924818	MF924886	MF924746	308
ZRCT18	MF924951	MF925019	MF925087	MF925159	MF924819	MF924887	MF924747	297
ZRCT19	MF924952	MF925020	MF925088	MF925160	MF924820	MF924888	MF924748	274
ZRCT20	MF924953	MF925021	MF925089	MF925161	MF924821	MF924889	MF924749	313
ZRCT21	MF924954	MF925022	MF925090	MF925162	MF924822	MF924890	MF924750	304
ZRCT22	MF924955	MF925023	MF925091	MF925163	MF924823	MF924891	MF924751	277
ZRCT23	MF924956	MF925024	MF925092	MF925164	MF924824	MF924892	MF924752	309
ZRCT24	MF924957	MF925025	MF925093	MF925165	MF924825	MF924893	MF924753	296
ZRCT25	MF924958	MF925026	MF925094	MF925166	MF924826	MF924894	MF924754	278
ZRCT26	MF924959	MF925027	MF925095	MF925167	MF924827	MF924895	MF924755	270
ZRCT27	MF924960	MF925028	MF925096	MF925168	MF924828	MF924896	MF924756	411
ZRCT28	MF924961	MF925029	MF925097	MF925169	MF924829	MF924897	MF924757	279
ZRCT29	MF924962	MF925030	MF925098	MF925170	MF924830	MF924898	MF924758	290
ZRCT30	MF924963	MF925031	MF925099	MF925171	MF924831	MF924899	MF924759	307
ZRCT31	MF924964	MF925032	MF925100	MF925172	MF924832	MF924900	MF924760	289
ZRCT32	MF924965	MF925033	MF925101	MF925173	MF924833	MF924901	MF924761	284
ZRCT33	MF924966	MF925034	MF925102	MF925174	MF924834	MF924902	MF924762	280
ZRCT34	MF924967	MF925035	MF925103	MF925175	MF924835	MF924903	MF924763	278
ZRCT35	MF924968	MF925036	MF925104	MF925176	MF924836	MF924904	MF924764	283
ZRCT36	MF924969	MF925037	MF925105	MF925177	MF924837	MF924905	MF924765	311
ZRCT37	MF924970	MF925038	MF925106	MF925178	MF924838	MF924906	MF924766	275
ZRCT38	MF924971	MF925039	MF925107	MF925179	MF924839	MF924907	MF924767	412
ZRCT39	MF924972	MF925040	MF925108	MF925180	MF924840	MF924908	MF924768	273
ZRCT40	MF924973	MF925041	MF925109	MF925181	MF924841	MF924909	MF924769	301
ZRCT41	MF924974	MF925042	MF925110	MF925182	MF924842	MF924910	MF924770	279
ZRCT42	MF924975	MF925043	MF925111	MF925183	MF924843	MF924911	MF924771	303
ZRCT43	MF924976	MF925044	MF925112	MF925184	MF924844	MF924912	MF924772	300
ZRCT44	MF924977	MF925045	MF925113	MF925185	MF924845	MF924913	MF924773	279
ZRCT45	MF924978	MF925046	MF925114	MF925186	MF924846	MF924914	MF924774	302
ZRCT46	MF924979	MF925047	MF925115	MF925187	MF924847	MF924915	MF924775	279
ZRCT47	MF924980	MF925048	MF925116	MF925188	MF924848	MF924916	MF924776	45
ZRCT48	MF924981	MF925049	MF925117	MF925189	MF924849	MF924917	MF924777	293
ZRCT49	MF924982	MF925050	MF925118	MF925190	MF924850	MF924918	MF924778	282
ZRCT50	MF924983	MF925051	MF925119	MF925191	MF924851	MF924919	MF924779	288
ZRCT51	MF924984	MF925052	MF925120	MF925192	MF924852	MF924920	MF924780	320
ZRCT52	MF924985	MF925053	MF925121	MF925193	MF924853	MF924921	MF924781	299
ZRCT53	MF924986	MF925054	MF925122	MF925194	MF924854	MF924922	MF924782	292
ZRCT54	MF924987	MF925055	MF925123	MF925195	MF924855	MF924923	MF924783	306
ZRCT55	MF924988	MF925056	MF925124	MF925196	MF924856	MF924924	MF924784	310
ZRCT56	MF924989	MF925057	MF925125	MF925197	MF924857	MF924925	MF924785	311
ZRCT57	MF924990	MF925058	MF925126	MF925198	MF924858	MF924926	MF924786	285
ZRCT58	MF924991	MF925059	MF925127	MF925199	MF924859	MF924927	MF924787	316
ZRCT59	MF924992	MF925060	MF925128	MF925200	MF924860	MF924928	MF924788	277
ZRCT60	MF924993	MF925061	MF925129	MF925201	MF924861	MF924929	MF924789	269
ZRCT61	MF924994	MF925062	MF925130	MF925202	MF924862	MF924930	MF924790	294
ZRCT62	MF924995	MF925063	MF925131	MF925203	MF924863	MF924931	MF924791	305
ZRCT63	MF924996	MF925064	MF925132	MF925204	MF924864	MF924932	MF924792	276
ZRCT64	MF924997	MF925065	MF925133	MF925205	MF924865	MF924933	MF924793	289
CYCT1	MF924998	MF925066	MF925134	MF925138	MF924798	MF924866	MF924794	413

(*continued*)

Table 2
(continued).

No. of strains	SAPT1	SAPT2	SAPT3	SAPT4	LIP1	LIP4	ALS2	DST
CYCT2	MF924999	MF925067	MF925135	MF925139	MF924799	MF924867	MF924795	414
FXCT01	MF925000	MF925068	MF925136	MF925140	MF924800	MF924868	MF924796	415
FXCT02	MF925001	MF925069	MF925137	MF925141	MF924801	MF924869	MF924797	279

DST: Diploid sequence type based on multilocus sequence type analysis, SAPT: Secretory aspartyl proteinase tropicalis, LIP: Lipase.

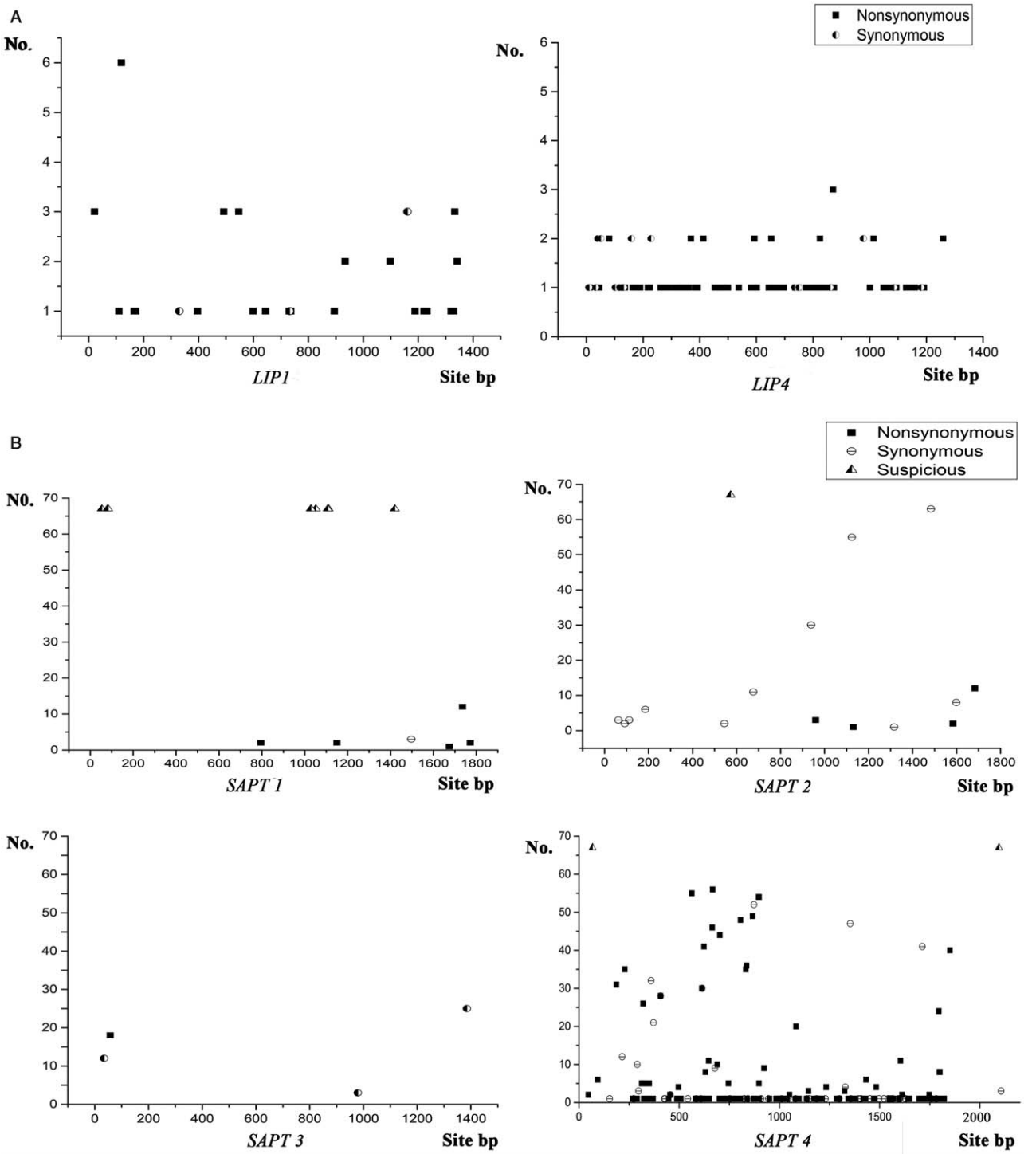

Figure 1: The number and location of SNPs present in *LIP1*, *LIP4*, and *SAPT1-4*. (A) The details of the SNPs in *LIP1* and *LIP4*. (B) The details of the SNPs in *SAPT1-4*. The horizontal axis represents the length of the genes. The vertical axis refers to the number of SNPs on each site and the location of SNPs on each gene. SNPs were analyzed to be either non-synonymous or synonymous mutations. *LIP*: Lipase, *SAPT*: Secretory aspartyl proteinase tropicalis, SNPs: Single nucleotide polymorphisms.

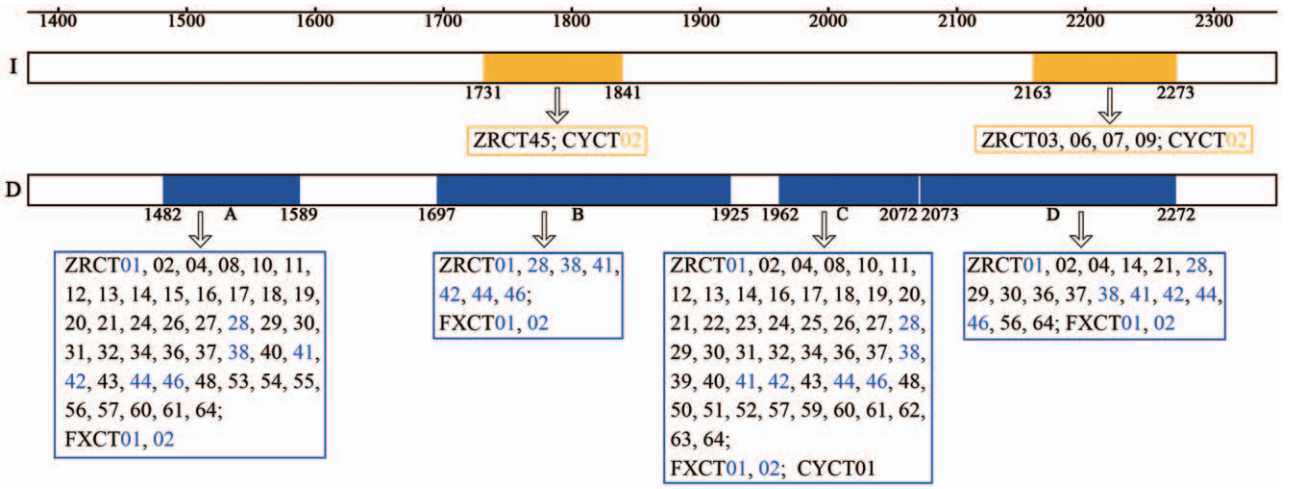

Figure 2: Insertions and deletions identified in *ALS2*. I: insertions on the pattern of *ALS2*. D: deletions on the pattern of *ALS2*. Orange refers to the location of insertions. Blue refers to the location of deletions. Names of isolates in the orange and blue square indicate the isolates with insertions and deletions, respectively. The orange and blue numbers in each square refer to the isolates with insertions and deletions in all sites. *ALS*: Agglutinin-like sequences.

located in the 1482 to 1589, 1697 to 1925, 1962 to 2072, and 2073 to 2272 bp regions, respectively [Figure 2]. There were more isolates with deletions than isolates with insertions. For isolates ZRCT01, 28, 38, 41, 42, 44, and 46, and FXCT01 and 02, deletions were detected in all 4 sites [Figure 2].

Three phylogenetic trees were constructed for each gene family, and they displayed specific evolutionary features and distinct gene diversities among individual isolates [Figure 3]. The results showed great heterogeneity in the *SAP* gene family and in *ALS2*, indicating possible microevolution among the isolates that correlate with stain-dependent characteristics. The *LIP* gene family was the most conserved as they almost diverged from the same origin at the same time, indicating no occurrence of microevolution among the tested isolates. Interestingly, no lipase activity was observed for the *C. tropicalis* isolates in our previous study.

Analysis of the hydrolytic enzymes (proteases, phospholipases, and hemolysins) of all isolates was published in our previous study.[10] All isolates produced protease and hemolytic activity, but none produced phospholipase and lipase activity. All isolates were divided into low, medium, and high activity groups. One strain each displayed high protease (ZRCT28), high hemolytic (ZRCT47), low protease (ZRCT64), and low hemolytic (ZRCT41) activities; the other isolates showed medium enzymes activities.[10] Adhesion and biofilm formation of these 68 isolates on polymethylpentene (PMP) and TCC-SUP cells were also performed in our previous study.[11] Strain ZRCT47 displayed the strongest adhesion and biofilm formation ability on both PMP and TCC-SUP surface as observed through crystal violet assay.[11] However, ZRCT45 exhibited the highest biofilm formation ability on TCC-SUP cells when XTT assay was performed.[11] Isolate FXCT01 obtained from blood had deletions on all 4 sites showed the lowest adhesion ability on PMP, and exhibited medium adhesion ability on TCC-SUP cells. Isolates with deletions in the 1697 to 1925 and 2073 to 2272 bp regions displayed relatively low abilities for adhesion and biofilm formation.

Discussion

Adhesion, biofilm formation, and hydrolytic enzyme activity were recognized as the key pathogenic elements of *Candida* species. In our previous study, the virulent phenotype of *C. tropicalis* was analyzed; we showed its strain-dependent features and the corresponding relationships with distinct genotypes.[12] In this study, the complete sequences of virulence-related genes were obtained and their correlations with phenotype were analyzed.

It is known that *Candida* species have several different adhesins (special cell wall proteins) that allow adhesion to specific substrates. Agglutinin-like sequence (ALS) proteins are an important family of proteins involved in the process of adhesion by mediating attachment to different epithelial cells and functioning as an adhesin.[13] Furthermore, Southern blot analysis with ALS-specific probes suggested at least three ALS-encoding genes in *C. tropicalis*,[14] but no further work has been undertaken in this area. Limited information regarding the DNA sequences of *ALS* was found in GenBank. Moreover, the whole-genome sequence of *C. tropicalis* revealed 16 *ALS*-like sequences.[4] From the sequences deposited in GenBank, we successfully amplified the whole length of the gene *ALS2*. The heterogeneity of *ALS2* was the strongest among the 16 *ALS*-like sequences. Furthermore, deletions occurred more frequently than insertions in *ALS2*. For the isolates obtained from blood (FXCT01 and 02), deletions were found in all four sites [Figure 2], and this might explain their low adhesion abilities. Interestingly, isolates with deletions located in the 1697 to 1925 and 2073 to 2272 bp regions showed lower adhesion and biofilm formation abilities on PMP. Functional analysis was needed to more deeply explore the relationship between *ALS2* and the adhesion ability of *C. tropicalis*.

The Saps of *Candida* have been intensively investigated.[15] The secretion of Sap1-10 by *C. albicans* is recognized as an

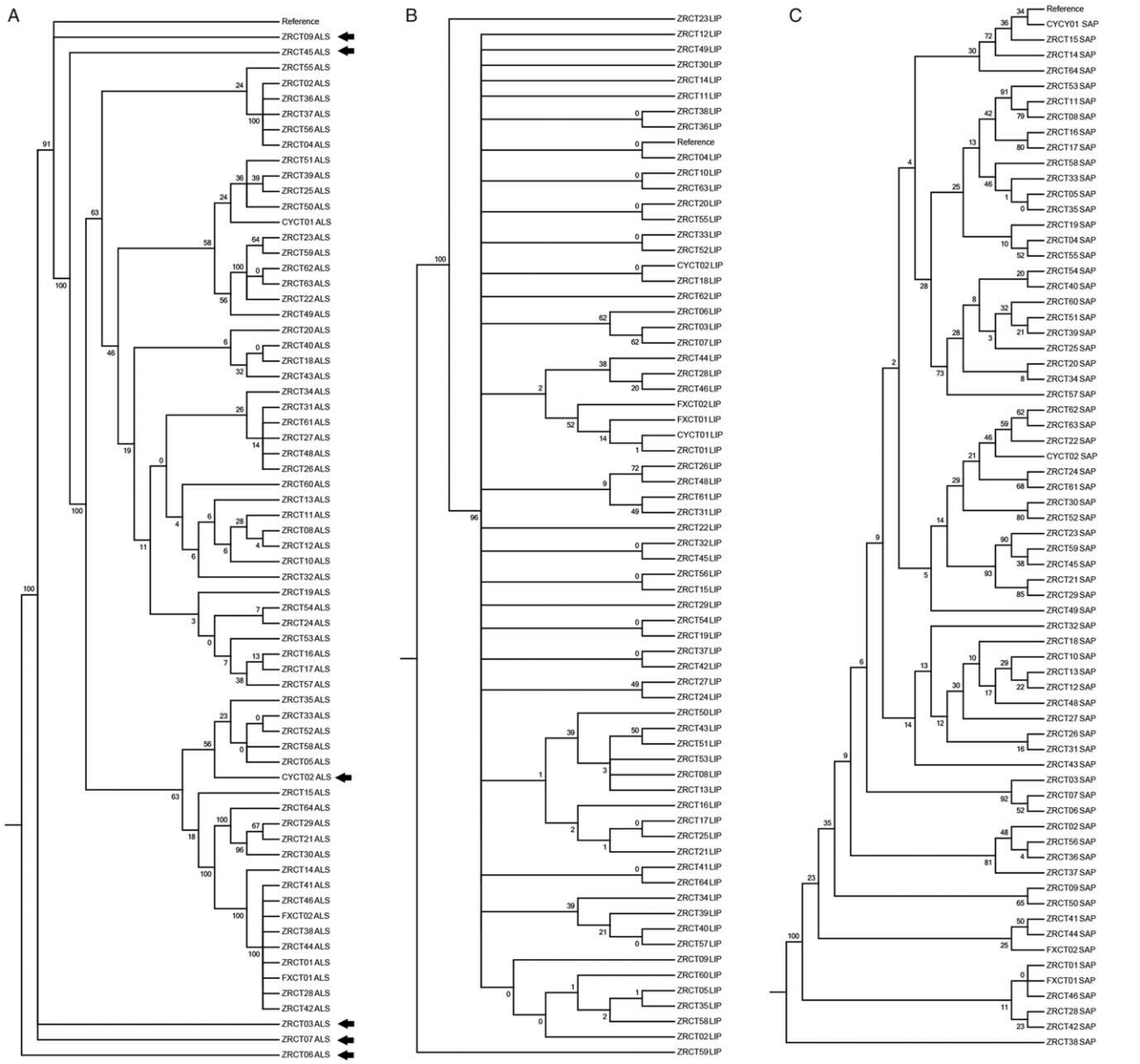

Figure 3: Neighbor-joining phylogenetic trees based on concatenated *LIP*, *SAPT*, and *ALS2* nucleotide sequences. (A) Phylogenetic tree based on *ALS2*. (B) Phylogenetic tree based on *LIP*. (C) Phylogenetic tree based on *SAPT*. Arrows refer to isolates with insertions within gene *ALS2*. *ALS*: Agglutinin-like sequences; *LIP*: Lipase; *SAPT*: Secretory aspartyl proteinase tropicalis.

important virulence determinant for this species.[16] Saps facilitate the colonization and invasion of host tissues through the disruption of host mucosal membranes, as well as by degrading important immunological and structural defense proteins.[17] A total of 4 *SAPT* gene families of *C. tropicalis* were identified; however, *SAPT1p* is the only 1 that has been purified from culture supernatant, biochemically characterized, and crystallized.[18] Sap secretion by *C. tropicalis* has also been detected when they penetrate tissues during disseminated infection, as well as on macrophages following phagocytosis of yeast cells.[19] The *SAP* genes of *C. tropicalis* have been studied widely; however, complete sequences available on GenBank were limited. Here, we acquired the whole length of *SAPT1-4* and found that genes *SAPT1-3* were relatively conserved, while *SAPT4* had great diversity. It is known that *SAPT2* and *SAPT4* were included in the multilocus sequence typing scheme of *C. tropicalis*. No significant relationship between gene sequence and activity of Saps was found in this study.

In addition, lipases (LIPs), which are involved in both the hydrolysis and synthesis of triacylglycerols, are often considered to be involved in *C. tropicalis* pathogenicity and contributes to host cell membrane damage.[20] Ten genes encoding LIPs (*LIP1-10*) have been identified in *C. albicans*, and 5 similar lipase-encoding genes were also detected in *C. tropicalis*.[21] Based on partial sequences and

the whole genome of *C. tropicalis* obtained from GenBank, *LIP1* and *LIP4* were successfully amplified. Although *LIP1* displayed more diversity than *LIP4*, no lipase activity was found in all 68 *C. tropicalis* isolates.[22-25]

In conclusion, we amplified and sequenced the complete lengths of *ALS2*, *LIP1*, *LIP4*, and *SAPT1-4* of 68 clinical *C. tropicalis* isolates. SNPs and indels were found in these genes, and these were phenotypically analyzed; deletions found in *ALS2* were associated with low adhesion ability. This study might be useful for further exploration of gene function in *C. tropicalis*. These findings should be verified using more isolates and the function of these genes should be studied further.

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Conflicts of interest

None.

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