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Evolution of GPI-Aspartyl Proteinases (Yapsines) of *Candida* spp

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1. Introduction

The *Candida* genus is a polyphyletic genus with at least 150 species. Nine are recognized opportunistic pathogens of humans and animals. *C. albicans* is the species most frequently isolated from human infections, followed by Candida non-Candida species (CNCA), as *C. glabrata*, *C. tropicalis*, C. *dubliniensis*, *C. parapsilosis*, *C. guilliermondii*, *C. lusitaniae, C. kefyr* and *C. krusei* (Méan et al. 2008; Pfaller & Diekema, 2007; Almirante et al. 2005; Manzano-Gayosso et al. 2000).

Some works describe the phylogenetic relationships of *Candida* genus and illustrate the limited relationship between the pathogenic *Candida* spp. The genus has been divided into: the CTG clade, which includes yeast that encodes CTG as serine instead of leucine (*C. albicans, C. dubliniensis, C. tropicalis, C. parapsilosis* and *C. lusitaniae*); and the WGD clade, which includes yeast that has undergone a genome duplication event (*Saccharomyces* spp., *Kluyveromyces* spp. and *C. glabrata*). Evidently, *C. glabrata* is more related to non-pathogenic yeasts, as *Saccharomyces cerevisiae*, than to the other pathogenic species (Scannell et al. 2007).

C. albicans is a normal microorganism in humans, and colonise up to 70% of skin, mucoses, and faeces of individuals with no apparent detriment to health. However, in some circumstances, either through environmental factors or a weakening of the host immune system, a proliferation and infection by *C. albicans* arise inducing candidosis (Wei et al. 2011).

Biofilm formation, adhesion, cavitation, phenotypic switching, dimorphism, interaction with the host immune system, invasion and tissue damage are virulence virulence factors for *C. albicans*. All these factors are related to the secreted aspartyl proteases (Sap) family, which is considered an important virulence factor and is studied as a possible target for therapeutic drug design (Naglik et al. 2004; Chaffin et al. 1998; Hube, 1998; Naglik et al. 2003, 2004, 2008).

The topic of this chapter is to understand the molecular characteristics, evolution and putative functions of glycosylphosphatidylinositol (GPI)-linked aspartyl proteases (Yps), a protein superfamily distributed among all pathogenic *Candida* species. Cell location motifs,

gene duplications, similitude, synteny, putative transcription factor binding sites and genome traits of the Yps family members are analysed by bioinformatics tools in an evolutionary context.

2. Aspartyl proteases

Aspartyl proteases or acid proteases (optimum activity at acidic pH) are proteins with a signal peptide in the amino-terminal site, at least one aspartic residue in the active site, and 4 cysteins (Hube & Naglik, 2001). The signal peptide is processed in the endoplasmic reticule and the protein is transported to their corresponding cell localization by the secretory pathway. The active site is formed by different amino acids. The consensus pattern described by PROSITE-EXPASY (http://expasy.org/prosite/) is [LIVMFGAC]-[LIVMTADN]-[LIVFSA]-D-[ST]-G-[STAV]-[STAPDENQ]-{GQ}-[LIVMFSTNC]-{EGK}-[LIVMFGTA], and the cysteins help the protein to the three dimensional structure by intramolecular disulfide bond (Fig. 1). According to the cell localization, aspartyl proteases could be secreted, or destined to vacuole or cell membrane by a GPI-linked site in the carboxyl-terminal residues (Alberch et al. 2006; Jones, 1991; Naglik et al. 2003).

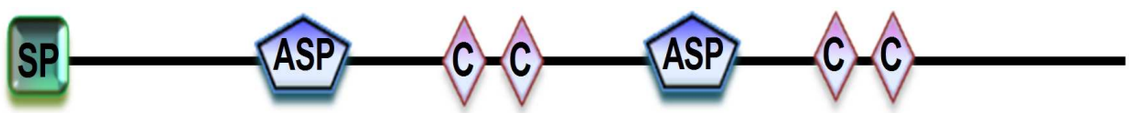

Fig. 1. Typical molecular structure of aspartyl proteases. SP: signal peptide; ASP: aspartic residue in the active site, C: cystein.

2.1 Secreted aspartyl proteases (Sap)

The *C. albicans* secreted aspartyl protease family comprises ten members, eight of which are proper secreted Sap1-Sap8, and two, Sap9 and Sap10, that have been reclassified as GPI-anchored aspartyl proteases (Alberch et al. 2006). Nevertheless, Sap9 and Sap10 are clearly more phylogenetically related to Sap than any GPI-anchored aspartyl proteases (Parra et al. 2009). The function of Sap in *C. albicans* has been widely studied, and these proteases are important in proteolysis to get a source of nitrogen, and are differentially regulated depending on the environmental conditions (Schaller et al. 1998; 2003; Taylor et al. 2005; Naglik et al. 2008). *SAP1*-SAP3 are relevant in phenotypic switching during the opaque phase and are not expressed in the WO-1 phase (Morrow et al. 1992; White et al. 1993). Also, they are expressed when yeast colonize and damage reconstructed human epithelium, oral and vaginal, which means that these Sap are important in superficial infections (Schaller et al. 1998; 2003; Copping et al. 2005). *SAP1-SAP8* are related to tissular damage (Taylor et al. 2005). *SAP1*, *SAP3* and *SAP8* are expressed in oral and vaginal infections. On the other hand, *SAP4-SAP6* are related to systemic infections and only they are expressed in yeast and germ tube at pH 5-7 (Hube et al. 1997; Sanglard et al. 1997; White & Agabian, 1995). Meanwhile *SAP5* is important in epithelial colonization, invasion and infection (Naglik et al. 2008; Lermann & Morschhäuser, 2008).

This kind of proteases are no exclusive of *C. albicans*. Orthologous genes have been described in other closely related species, as *C. dubliniensis* (Sap1-4 and Sap7-10), *C. tropicalis* (Sapt1-12), *C. guilliermondii* (Sapg1-8), *C. parapsilosis* (Sapp1-14) and *C. lusitaniae* (Sapl1-3)

(Parra et al. 2009). Particularly in *C. dubliniensis*, the expression of *SAPD3* and *SAPD4* genes is related to the infection of keratinocyte (HaCAT cells) by yeast. The number and shape of the keratinocyte cells was altered by the infection, but these effects decreased in the presence of pepstatin A, an aspartyl protease inhibitor, suggesting that the Sapd3 and 4 of *C. dubliniensis* could be considered as virulence factors, like their orthologous genes from *C. albicans* (Loaiza-Loeza et al. 2009). The function of these proteases in metabolism and pathogenesis in the rest of pathogenic species is unknown.

According to Dayhoff, protein superfamilies and families are defined as groups of related proteins that exhibit less than 50% and greater than 50% similarity, respectively. Subfamilies were defined as groups of proteins with at least 90% similarity and were often equivalent to clusters of orthologous groups (COGs) (Dayhoff, 1979). Behind this idea, the phylogeny of pathogenic *Candida* spp. Saps allows for the recognition of a superfamily with at least 12 paralogous families and nine orthologous subfamilies. In several Sap families, at least two subfamilies or orthologous groups are proposed (Parra et al. 2009).

2.2 Vacuolar aspartyl proteases (PrA)

The vacuole is a hydrolytic organelle similar to lysosomes in animals and is the site of non-specific degradation of cytoplasmic proteins (Robinson et al. 1988), proteins delivered via autophagy (Klionsky & Emr, 2000), or plasma membrane proteins turned over via endocytosis (Hicke, 1996). In *S. cerevisiae* the vacuole has been studied and possesses different vacuolar proteases (Table 1).

One of the most important vacuolar proteins is the proteinase A (PrA), encoded by the *PEP4* gene. Mutants in *PEP4* (*pep4*) accumulate multiple zymogens, indicating that PrA initiates processing, maturation and activation of multiple different precursors of PrB, DAP, CPY and PrA, because of their autocatalytic activity and their lack of production of dead cells in nutritional stress. Also, PrA is important in cellular response to starvation, microautophagy, proteolysis involved in cellular and vacuolar protein catabolic process, and sporulation (Palmer, 2007; Jones, 1991; Teichert et al. 1989).

The function of PrA, encoded by the *CaPEP4* gene in the metabolism of *C. albicans*, has also been studied. Null mutants of *CaPEP4* maintain their hydrolytic activity intact, clearly suggesting that *C. albicans* possesses an alternative system that compensates for the lack of this gene (Palmer, 2007). In *C. albicans*, the vacuole is important in cell differentiation, surviving into macrophages, and elimination of drugs as hygromicin B, orthovanadate and rapamicine (Palmer, 2005).

In *C. dubliniensis*, this protein could be important in carbon and nitrogen metabolism and might participate in protein degradation and precursor processing as occurs in *S. cerevisiae* (Loaiza et al. 2007). The genome-wide environmental stress response expression profile of *C. glabrata* revealed that *CgPEP4* is induced in osmotic stress and glucose starved conditions. Meanwhile, in *S. cerevisiae* no changes in the expression were observed in the same conditions (Gash et al. 2000; Roetzer et al. 2008).

Bioinformatic genomic analysis of *Candida* pathogenic species exhibited that only one version of PrA is harboured by yeast (Table 3), but apparently the *CgPEP4* gene is universally distributed among *C. glabrata* strains, as revealed by PCR multiplex in a collection of 52 *C. glabrata* clinical strains (Table 5; Fig. 3; for PCR conditions see 2.3 section). Phylogenetic analysis was performed by an aligment of PrA homologues identified *in silico* and those previously characterized. The aligment was conducted using MUSCLE in SeaView 2.4 program (Galtier et al. 1996) with default alignment parameter adjustments.

The phylogenetic analyses were performed in the MEGA4 program (Tamura et al. 2007) using Maximun Parsimony evolution. A similitude and identity matrix were computed with the MatGAT4.50.2 software (Campanella et al. 2003). The phylogenetic reconstruction and similarity of PrA reproduce the phylogenetic tree topologies of *Candida* spp. obtained with other genes, suggesting a common ancestral gene (Fig. 2; Table 2). In brief, *C. albicans* was more related to *C. dubliniensis*, followed by *C. tropicalis*, *C. parapsilosis*, *C. guilliermondi* and *C. lusitaniae*. Meanwhile, *C. glabrata* PrA was more related to *S. cerevisiae* PrA than other *Candida* species.

Name/systematic name	Gene/ Protein	Access number	Function	Reference
Proteinase A YPL154C	PEP4/ PrA	NM_001183968 / NP_015171	Activities of other yeast vacuolar hydrolases	Parr et al., 2007
Carboxypeptidase Y YMR297W	CPY	NM_001182806 /NP_014026	Contributes to the proteolytic function of the vacuole	Wünschmann et al., 2007
Proteinase B YEL060C	PRB1	NM_001178875 /NP_010854	Involved in protein degradation in the vacuole and required for full protein degradation during sporulation	Teichert et al., 1989
Carboxypeptidase S YJL172W	CPS	X63068/ CAA44790	Nitrogen compound metabolic process, proteolysis involved in cellular protein catabolic processes	Bordallo & Suarez-Rendueles, 1993
Dipeptidyl aminopeptidase B* YHR028C	DAP-B	X15484/ CAA33512	Protein processing	
Aminopeptidase YKL103C	APEI	NM_001179669 /NP_012819	Catabolic processes	

Table 1. Soluble and membrane-bound * vacuolar proteolytic system of *S. cerevisiae*.

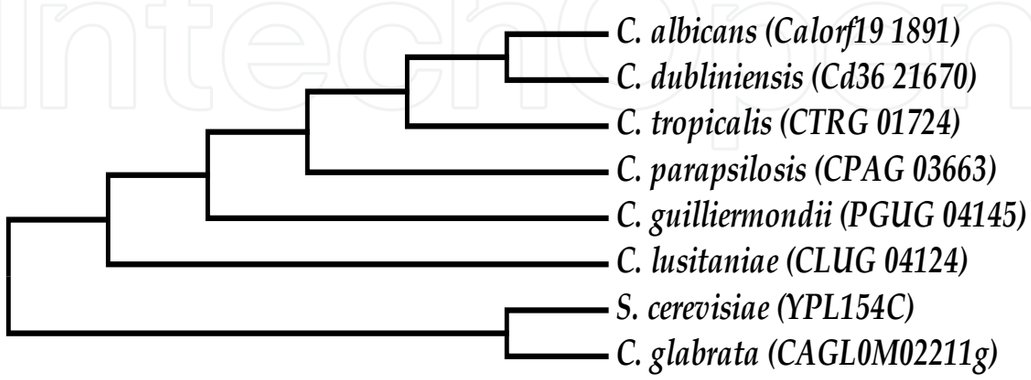

Fig. 2. Maximun Parsimony phylogenetic analysis of vacuolar aspartyl proteases (PrA) superfamily from pathogenic *Candida* spp.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1. *S. cerevisiae* **YPL154C**		62	66	66	66	66	65	68
2. *C. glabrata* **CAGL0M02211g**	77		55	55	54	54	55	57
3. *C. albicans* **orf19_1891**	77	69		98	90	85	75	78
4. *C. dubliniensis* **Cd36_21670**	77	69	99		91	85	76	79
5. *C. tropicalis* **CTRG_01724**	76	69	97	97		86	75	77
6. C. *parapsilosis* **CPAG_03663**	76	67	91	92	93		74	78
7. *C. guilliermondii* **PGUG_04145**	79	69	84	85	85	85		78
8. *C. lusitaniae* **CLUG_04124**	78	69	86	86	87	86	87	

Table 2. Similarity and identity (UP/down) between PrA proteins from pathogenic *Candida* spp.

PrA (AN)	**Amino acid residues**	**MM (kDa)**	**IP**	**MOTIF**	**Signal peptide (aa)**	**C**
C. albicans Calorf19_1891	419	45.4	4.5	119-130: VILDTGSSNLWV 304-315: AAIDTGTSLITL	20	7
C. dubliniensis Cd36_21670	419	45.4	4.5		14	2
C. tropicalis CTRG_01724	422	45.6	4.5	121-132: VILDTGSSNLWV 306-317: AAIDTGTSLITL	24	2: 1400605-1401870-
C. parapsilosis CPAG_03663	428	46	4.5	127-138: VILDTGSSNLWV 312–323: AAIDTGTSLITL	25	130: 135636-136919 -
C. guilliermondii PGUG_04145	409	44	4.3	109-120: VILDTGSSNLWV 294-305: AAIDTGTSLITL	21	5: 355498-356724 -
C. glabrata CLUG_04124	407	43	4.3	107-118: VILDTGSSNLWV 292–303: AAIDTGTSLITL	19	5: 46984-48204 -

Table 3. Vacuolar aspartyl proteases in pathogenic *Candida* species. (AN): Access number in the respective genome; MM: molecular mass; IP: Isoelectric Point; C: Chromosome or Contig or supercontig.

C. glabrata is an opportunistic haploid yeast that suffered evident and extensive reductive evolutionary events. A lot of genes involved in nitrogen metabolism, carbohydrate assimilation (saccharose, galactose, etc.), as well as sulfur, phosphor, thiamine, pyridoxine and nicotinic acid biosynthesis have been lost from the genome (Byrne & Wolfe, 2005;

Wolfe, 2006). This species produces between 15-20% of reported systemic yeast infections (Almirante et al. 2005; Manzano-Gayosso et al. 2000; Trick et al. 2002; Méan et al. 2008). *C. glabrata* is the most common yeast species isolated from patients with cancer, organ transplantation and fluconazole therapy (Safdar et al. 2001; Bodey et al. 2002). The mortality associated with *C. glabrata* in systemic infections of cancer patients is 50% and almost 100% in transplant patients (Anaissie et al. 1992; Goodman et al. 1992; Krcmery et al. 1998). This scenario is related to indiscriminate antifungal use, and to the innate resistance of *C. glabrata* (Sobel, 2006).

According to Table 4, virulence factors of *C. albicans* and *C. glabrata* are quite different. However, an evident feature is the difference in number and kind of aspartyl proteases. A total of 12 *YPS* genes, but no *SAP* genes have been detected in *C. glabrata.* Contrarily, a total of 10 *SAP* genes, but no *YPS* genes have been recognized in *C. albicans.* Clearly, the phylogenetic trees constructed with ribosomal or other gene groups include the majority of the clinical relevant *Candida* species, with exception of *C. glabrata*, which is grouped in another cluster with non-pathogenic yeasts, as *S. cerevisiae* and *Kluyveromyces* spp. This evidence suggests that the aspartyl proteases in *Candida* spp. have evolved independently as virulence factors at least two times, and possibly the amplification by duplication of *SAP* and *YPS* gene superfamilies in clinically relevant species is an example of convergent evolution.

A physiological approach could possibly contribute to the understanding of which *C. glabrata YPS* (*CgYPS*) genes are covering the functions of each secreted aspartyl protease of *C. albicans* under different conditions. Evidently, the comparison of virulence strategies, expression profiles, complementation of mutants, among other experiments, could suggest common and particular features and roles for all *SAP* and *YPS* genes. For now, the questions remain open. Have the function of *C. glabrata CgYPS* and *SAP C. albicans* genes functionally converged?

The transcription profile of 11 *CgYPS* was studied when yeast were ingested by macrophages. Apparently, *CgYPS* are important in survival and virulence of the the yeast in macrophages, damage to mousses, Epa1 protein processing, and cell wall integrity, as occur in *S. cerevisiae*, which possesses *5 ScYPS* (*ScYPS1-ScYPS3, ScYPS6* and *ScYPS7*) (Kaur et al. 2007; Krysan et al. 2005). They are important to cell wall synthesis and glucan homeostasis, mainly *ScYPS1* and *ScYPS7*. It seems that *ScYPS3* does not have functions associated with the cell wall (Krysan et al. 2005).

C. albicans SAP9 and *C. glabrata CgYPS1* genes complement the defects in the cell wall provoked by *yps1*Δ of *S. cerevisiae*. One important difference is that *SAP9* complement *yps1*Δ only when *SAP9* is under a heterologous and constitutive promoter from *S. cerevisiae*, while *CgYPS1* complements the mutation, using its promoter (Krysan et al. 2005), evidence that supports the othologous status proposed above for these gene pairs. As happened with *ScYPS1, SAP9* gene expression increases during the stationary phase and damage of the cell wall (Monod et al. 1998; Copping et al. 2005), and protects the yeast from caspofungin (an inhibitor of β–1,3-glucan synthesis) (Lesage et al. 2004). Also, inhibitors of ScYps1p disable the specificity of both proteins, ScYps1p and Sap9 (Cawley et al. 2003).

Distribution of the *SAP* gene superfamily among *C. albicans* strains is universal (Gilfillan et al. 1998; Bautista et al. 2003; Parra et al. 2009), although one study concludes that the distribution of *SAP* genes in clinical strains depends on infection associated with isolation

(Kalkanci et al. 2005). Given the number of *CgYPS* in *C. glabrata* and their potential role in pathogenesis, it is important to establish the universality of *CgYPS* in *C. glabrata* populations.

Factor	*C. glabrata*	*C. albicans*	**Reference**
Infection sites	Oral, vaginal, bloodstream		Fidel et al. 1999
Mortality in systemic infection	urinary tract		Abi-Said et al. 1997; Krcmery, 1999
Virulence in animal models	High		Arendrup et al. 2002
Filamentation	Present		Lachke et al. 2002; Csank & Haynes, 2000
Biofilm formation	High		Castaño et al. 2006
Adherence to oral keratinocytes	Lower	Higher	Nikawa et al. 1995; Biasoli et al. 2002
Adherence to denture material	Lower	Higher	Luo & Samaranayake, 2002
Extracellular proteinase activity	Absent	Present	Chakrabarti et al. 1991
Phospholipase activity	Isolation site dependent	High	Samaranayake et al. 1994; Ghannoum, 2000
Phenotypic switching	Low	High	Brockert et al. 2003
IL-8 induction in oral keratinocytes	Pseudohyphae	True hyphae and pseudohyphae	Schaller et al. 2002
GM-CSF induction in oral keratinocytes	Weak	Strong	Schaller et al. 2002; Li et al. 2007a
Human-defensin resistance	Strong	Weak	Joly et al. 2004; Feng et al. 2005
Histatin resistance	Partially resistant	Susceptible	Helmerhorst et al. 2005
Azole resistance	High	Low	Sanglard et al. 1999
Molecules involved in adherence	20 *EPA* genes	*ALS* proteins	Castaño et al. 2005; Hoyer et al. 2001
SAP genes	0	10	Parra et al. 2009
YPS genes	12	0	Albrecht et al. 2006; Kaur et al. 2007 This work

Table 4. Comparison of virulence factors of *C. glabrata* and *C. albicans* (modified from Li, 2007b).

Our group explored the *CgYPS* gene distribution among clinical isolates (n=52) and type strains CBS138 and BG6 (N=2) by an original multiplex PCR procedure (Table 5). The yeasts were routinely grown on YPD broth and DNA was extracted using a previously reported protocol (Hoffman & Winston 1987). PCR was performed in a DNA thermal cycler 9600

(Applied Biosystems, Foster City, CA). Amplification reactions (25 µL) were performed using a buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.6 µM each primers, 4 ng/µL of genomic DNA, and 1.5 U/µL of *Taq* polymerase (Invitrogen). The PCR conditions included a denaturation step for 3 min at 94°C, followed by 38 amplification cycles consisting of 1 min at 94°C, 1 min annealing temperature and 1 min at 72°C. A final extension step was performed for 7 min at 72°C. Fig. 3 shows the amplification products of *CgYPS* gene fragments of some representative *C. glabrata* clinical strains electrophoresed in 1% agarose gels. Similar PCR conditions were used to study the universal distribution of PrA.

Gene	Primer	Location	Expected amplified fragment (bp)	Tm (°C)
CgYPS1 CAGL0M04191g	F:5´-TTCTGGTGACAGTTGTATCTTGG-3´ R:5´-GATAAATGAAACCAAAAGACCAGCG-3´	+1326 a +1348 +1779 a +1803	477	55
CgYPS2 CAGL0E01419g	F:5´-ACTCAACTTGTTTTTAACTTCGGTGGTGC-3´ R:5´-TAGCATGGAGAGTAGGATGTTAAACACC-3´	+1234 a +1262 +1743 a +1770	536	61
CgYPS3 CAGL0E01727g	F:5´-AAAGCAAGTCGTCGATGTCATCG-3´ R:5´-TTGCAACTAACACTAAAGTGGTGC-3´	+951 a +973 +1580 a +1603	652	58
CgYPS4 CAGL0E01749g	F:5´-TTCTGTGTTACCAGCAAAGGTTGC-3´ R:5´-TTAATGTAGTTCTCTTACGGAGAGC-3´	+933 a +956 +1411 a +1435	502	55
CgYPS5 CAGL0E01771g	F:5´-TATACATATATGCCAAGCAGCGTTGC-3´ R:5´-AACAAGGCAGTAACTGCTGATAAAGC-3´	+934 a +959 +1528 a +1553	619	58
CgYPS6 CAGL0E01793g	F:5´-ACCAGAAGGTAGCTGCATTAATCG-3´ R:5´-AATGGTAGCTAATATGGCAGCAACG-3´	+887 a +910 +1542 a +1518	631	58
CgYPS7 CAGL0A02431g	F: 5´-TATGGGACCAATCTATATAACGTCC-3´ R: 5´-TAAGTAGCATACGGTATGTAGCCC-3´	+831 a +855 +1404 a +1427	596	55
CgYPS8 CAGL0E01815g	F: 5´-TTGGGATTACAGGGTAATGATGC-3´ R: 5´-AACTCTTTTTTGAAGGTCAAAACGCG-3´	+856 a +878 +1457 a +1482	626	58
CgYPS9 CAGL0E01837g	F: 5´-TTCCGTAAATGTGACTGATTTCATGG-3´ R: 5´-ATCATAATGAGTATGGCAGAGTTGGC-3´	+1071 a +1096 +1510 a +1535	464	58
CgYPS10 CAGL0E01859g	F: 5´-TAATAAGACGGAAGCCATCAGACTGC-3´ R: 5´-TTGTAATTGCTGCTAGTACTAGGACG-3´	+978 a +1003 +1479 a +1504	526	58
CgYPS11 CAGL0E01881g	F: 5´-TTGGTGTCCCATACAAGGAAATGGTC-3´ R: 5´-AATCCACAAG ACCAGCAACA GGATAGC-3´	+1100 a +1125 +1495 a +1521	421	61
CgYPS12 CAGL0J02288g	F: 5´-AATTGCACATGAAGATTCCGTTGCG-3´ R: 5´-TATCAGTTATTGTAGCAGTTACTGGC-3´	+1001 a +1025 +1542 a +1567	566	58
CgPEP4 CAGL0M02211g	F 5´ -TATCTGAAGAGTGTCAATGACCCAGC-3´ R 5´-TACAGCCTCAGCTAAACTGACAACATTGG-3´	+691 - +716 +1208 - +1236	545	58

Table 5. Primer pairs used for conventional multiplex PCR of *C. glabrata YPS* genes. Bp, Base pair; Tm, Melting temperature.

The universality of the 12 *CgYPS* genes among all *C. glabrata* clinical isolates and type strains was confirmed (Fig. 3), which suggests that all *CgYPS* are important to yeast life cycle as pathogen or commensal, and probably are differentially regulated according to each environmental condition, as occurs with *C. albicans SAP*.

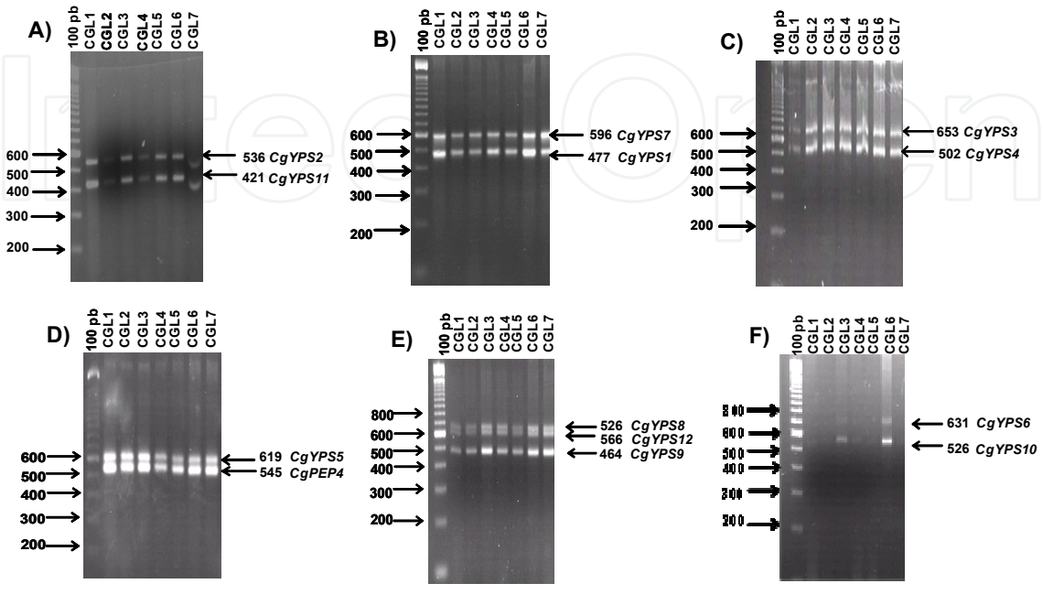

Fig. 3. Amplification of *CgYPS C. glabrata* gene fragments by multiplex PCR. A, *CgYPS2* and *CgYPS11*; B, *CgYPS7* and *CgYPS1*; C, *CgYPS3* and *CgYPS4*; D, *CgYPS3* and *CgPEP4*; E, *CgYPS8, CgYPS12* and *CgYPS9*; F, *CgYPS6* and *CgPEP10*.

2.3.1 *YPS genes* in clinically relevant *Candida* species

The genome sequence projects of *Candida* species allows for the exploration of whether *YPS* genes are harboured in these opportunistic pathogen yeasts. *C. dubliniensis* sequences were obtained from the Sanger Institute Microorganisms Sequencing Group (http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/). Sequences from *C. guilliermondii*, *C. lusitaniae*, *C. tropicalis* and *C. parapsilosis* were obtained from (http://www.broad.mit.edu/annotation/genome/candida_group/MultiHome.html). The GenBank database (http://www.ncbi.nlm.nih.gov) was also used. The detection was made by using the previous *YPS* and *SAP* genes detected in *S. cerevisiae* (http://www.yeastgenome.org), *C. glabrata* (http://cbi.labri.fr/Genolevures/elt/CAGL) and *C. albicans* (http://www.candidagenome.org) genomes, and the proteins detected by BLAST analysis in NCBI. Also, the different patterns of motif that could be obtained were used as a new query. In *C. lusitaniae* and *C. guilliermondii* only one *YPS* was detected. Meanwhile in *C. dubliniensis* and *C. albicans* four *YPS* genes were detected, in *C. tropicalis* two, and in *C. parapsilosis* six. Theoretical isoelectric point, molecular weight and amino acid content were calculated using Antheprot 2000 version 5.2 (Table 6).

Prediction of motif sequences was performed with PROSITE (http://www.expasy.org) (Falquet et al. 2002). Some of the proteins possess a typical molecular structure of aspartyl proteases, but others have some differences in composition (Fig. 4; Table 6). Some of them possess high Ser/Thr content in the amino terminal, suggesting that this zone is exposed at the surface of the protein. The presence of Ser/Thr in the carboxyl terminal in almost all *YPS*

is postulated to be heavily O-glycosylated. The exact function of this Ser/Thr-rich domain in yapsins has not been investigated. However, O-mannosylation is important for proper cell-wall biogenesis and integrity. It has also been proposed that clustered O-glycans create rigid stalks that keep protein domains away from membranes or wall surfaces (Lipke & Ovalle, 1998).

Yps (AN)	**Amino acid residues**	**MM (kDa)**	**IP**	**MOTIF**	**Signal peptide (aa)**	**C**
ScYps1 YLR120C	569	60	4.5	98-109: VLVDTGSSDLWI 368-379: ALLDSGTTLTYL	21	XVII
ScYps2 YDR144C	596	64.2	4.3	96-107: VLVDTGSSDLWV 356-368: VLLDSGTTISYM 496-570: SER	18	IV
ScYps3 YLR121C	508	54.5	8.4	78-89: VLLDTGSADLWV 285-296: ALLDSGTTLTYL 439-470: THR	20	XVII
ScYps6 YLR039C	537	58.2	3.9	82-93: LQLDTGSSDMIV 321-32: VMLDSGTTFSYL	24	IX
ScYps7 YDR349C	596	64.4	4.6	71-82: LLVD**VIIQ**PYINL 318-329: ALLDS**T**SSVSYL	16	IV
ScBar1 YIL015W	587			60-71: VLFDTGSADFWV 284-295: VLLDSGTSLLNA		
CgYps1 CAGL0M04191g	601	63.8	5.0	88-99: VLVDTGSSDLWI 375-386: ALLDSGTTLTYL	18	M
CgYps2 CAGL0E01419g	591	63.2	4.4	82-92: LLLDTGSSDMWV 366-377: ALLDSGTTVSYL	18	E
CgYps3 CAGL0E01727g	539	58.9	6.4	66-79: V**Q**LDTGSSDLWF 305-316: VLLDTGTTL**A**YA	14	E
CgYps4 CAGL0E01749g	482	53.2	8.4	65-76: V**Q**LDTGSSDLWF 303-14: **T**LLDTGVTTSVL	15	E

Yps (AN)	Amino acid residues	MM (kDa)	IP	MOTIF	Signal peptide (aa)	C
CgYps5 CAGL0E01771g	519	57.2	5.5	66-77: V**Q**LDTGSSDLWF 304-315: ALLDTGTTYTYM 60-70: LECT	15	E
CgYps6 CAGL0E01793g	516	55.9	4.6	65-76: V**Q**LDTGSADLWF 301-312: ALIDSGTTISEF 62-68: LECT	15	E
CgYps7 CAGL0A02431g	587	63.4	4.7	64-75: L**G**L**GLAQ**PYVWV 302-313: VLLD**PSF**ALSYL	18	A
CgYps8 CAGL0E01815g	519	56.7	6.8	65-76: V**Q**LDTGSSDLWF 304-315: ALLDSGTTLTVV	15	E
CgYps9 CAGL0E01837g	521	56.9	5.1	65-76: L**Q**IDTGSSDLFV 300-311: **T**LLDSGSTISLL	16	E
CgYps10 CAGL0E01859g	505	55.3	7.3	61-72: A**Q**LDTGSSDLWF 298-309: ALFDSGTSYSYV	13	E
CgYps11 CAGL0E01881g	508	55.6	5.0	63-74: LLVDTGSSDFWV 310-321: ALLDTGSTDTHL	29	E
CgYps12 CAGL0J02288g	541	59.5	4.6	68-79: LVLDTGSSDLWV 279-290: ALLDTGSTLI**E**L 448-495: SER	19	J
orf19_852	365	39.6	5.4	73-84: LAADTGS**W**LIQI 245-256: **Y**TIDTG**GR**Y**G**FL	17	2
orf19.6481	702	75.9	4.4	159-170: L**R**LD**LIQ**PEIWV 406-417: VLLDS**R**ASNFYL 565-662: SER	20	7

Yps (AN)	Amino acid residues	MM (kDa)	IP	MOTIF	Signal peptide (aa)	C
orf19_853	364	39.1	5.7	72-83: LSIDTGSWLTHI 244-255: **Y**TLDTG**GG**T**G**FL 42-44: RGD	17	2
orf19_2082*	436	47.7	3.8	53-64: VIVDSGSSDLMI 229-240: **YQ**IDSGTN**G**FV**P**	14	2
Cd36_18360				72-82: VVII-1DTGS**WLT**HI 848-859: **Y**TLDTG**GG**NGYL	17	2
Cd36_18370	365	40	5.6	73-84: IAADTGS**W**LT**QI** 246-257: **YTM**DTG**GG**Y**G**YL	17	2
Cd36_72090	697	76.6	4.6	149-150: LRLDLIQPEIWVM 402-412: VILDSRASNFY	13	7
Cd36_15430	442	48.7	4.2	60-71: VII-1VDSGSSDLMI 236-47: **YQ**IDSGTN**G**FV**P**	27	2
CTRG_05014	690	74.8	4	151-162: L**R**LD**LIQ**PEIWV 401-412: VLIDS**R**SS**Y**FYL	20	7: 407395-409464 -
CTRG_01112	432	47.9	3.8	55-66: VII-1VDSGSSDLMI 232-243: **YQ**IDSGSN**G**FL**P** 392-423: THR	20	2: 57814-59109 -
CPAG_04785	369	40.5	4.5	72-83: VMIDTGS**W**RLNV 245-256: I**G**IDSG**N**PRLAF	20	139:296423-297529 -
CPAG_04801	374	40.5	5.7	251-262: LALDTG**N**P**GIG**L 76-77: V**F**IDTGS**W**ALNF	19	139:334234-335355 -
CPAG_04802	371	40.4	6.1	76-87: VVII-1DTGS**W**ALNF 248-259:	19	139:337822-338934 +

Yps (AN)	Amino acid residues	MM (kDa)	IP	MOTIF	Signal peptide (aa)	C
				LAFDTGSA**G**LIL		
CPAG_03253	366	40.9	6.4	74-85: VLLDTA**S**TVLNV 246-257: VL**H**DSGTPTM**E**L	15	126: 70630-71727 +
CPAG_02564	366	40.5	6.5	74-85: VLLDTA**SI**VLNV 246-255: VL**H**DSGTPTMAL	15	116:138449 -139546 -
CPAG_04713	700	75.5	4.5	157-168: L**R**LD**LIQ**PEVWV 417-428: VLLDS**RIL**YSYL 19-55: SER	27	139:123872 -125971 +
PGUG_04882	583	63.5	4.1	81-92: LRLD**LTQ**PEIWV 224-235: LV**QQGVII**KSS**AY**	16	6: 496161-497909
CLUG_00903	722	74	3	63-74: VLLDTGSSDLWV 275-286: ALLDSGTSL**Q**YL 470-701: SER 540–638: THR	14	1: 1836367-1838532 +

Table 6. Aspartyl proteases GPI- linked to cell membrane in pathogenic *Candida* spp. AN: Access number in the respective genome; MM: molecular mass in kilodaltons (kDa); IP: Isoelectric Point; C: Chromosome/Contig or supercontig; the atypical amino acids in the PROSITE motif are shown in black (Eukaryotic and viral aspartyl protease active site).

The presence of a GPI attachment site, a characteristic feature of the yapsin family, was determined with big-PI predictor (http://mendel.imp.univie.ac.at/gpi/gpi_server.html), and GPI-SOM. GPI-anchor signals were identified by a Kohonen Self Organizing Map (http://gpi.unibe.ch/). A total of 36 protein sequences were analyzed, but GPI sites were recognized only in 21 proteins. GPI sites were not detected in ScYps2 and CgYps2, although both proteins have been previously confirmed as Yps proteins. The software programs must be enhanced, but an experimental approach to confirm the cell location is necessary.
PSORTII (http://www.psort.org/) and Softberry (http://www.softberry.com) programs were used to predict subcellular localization. All proteins detected seem to be extracellular, which could be because of the presence of a signal peptide in the amino terminal extreme. Nevertheless during their synthesis, yapsins are cotranslocated and modified by the addition of GPI to the lumen of the endoplasmic reticulum (ER). Then proteins are glycosylated in Golgi apparatus, associated to membrane vesicles and sent to plasma membrane or the cell wall (Mayor & Rieaman, 2004; Caro et al. 1997). Softberry program was also used to find exons, which were absent in all genes studied. A search was made for

internal protein sequence repeats to detect possible internal duplication events, but none were detected by TRUST (Szklarczyk & Heringa, 2004) even though it is likely were not

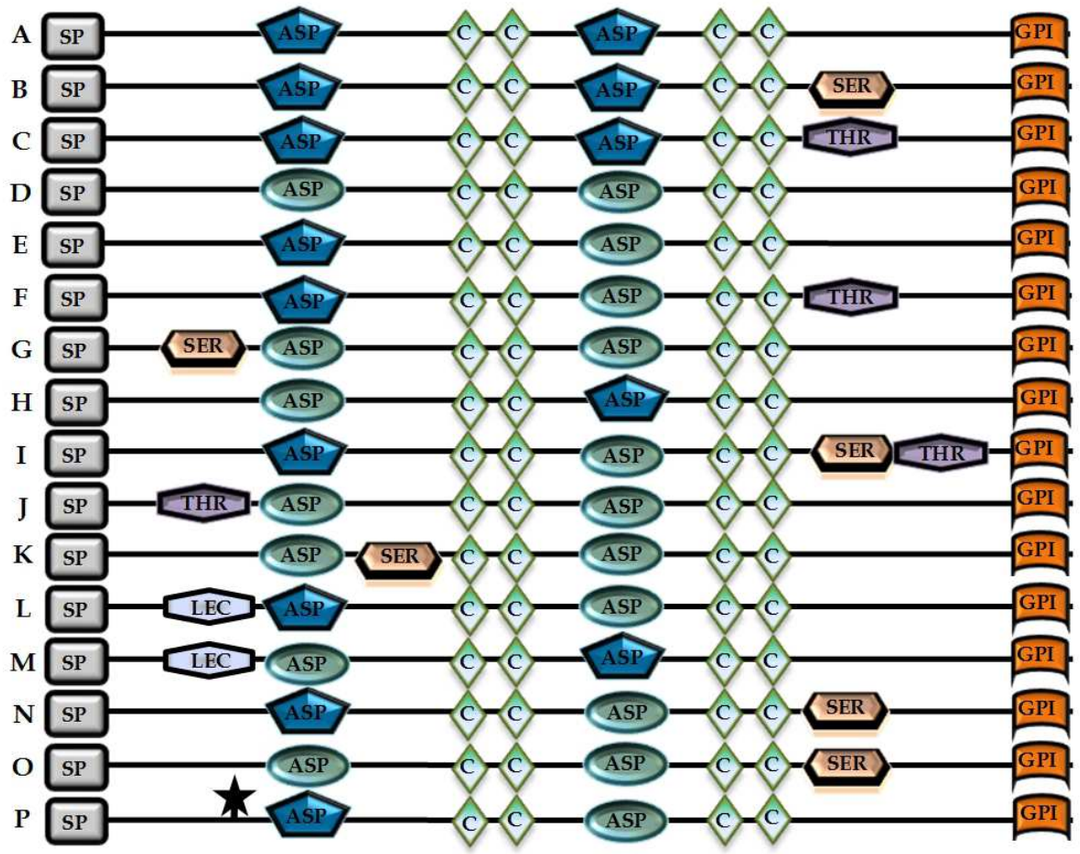

Fig. 4. Motifs of *Candida* spp. GPI-anchored aspartyl proteases (Yps). Rectangle boxes (SP): amine terminal signal peptide; pentagon (ASP): aspartyl protease domains in agreement with PROSITE; circles (ASP): atypical aspartyl protease domains proposed as [LIVMFGACTPSYF]-(LIVMTADNQSFH)-(LIVFSAE)-D-(STP)-(GS)-(STAV)-(STAPDENQY)-X-(LIVMFSTNCGQ)-(LIVMFGTAW); hexagons: serine (SER), threonine (THR), lecithin (LEC) rich regions; star: RGD motif; rhombus (C): cysteine residues, semicircles. Ca, *C. albicans*; Cd, *C. dubliniensis*; Cg, *C. glabrata*; Cgu, *C. guilliermondii*; Cl, *C. lusitaniae*; Cp, *C. parapsilosis*; Ct, *C. tropicalis;* Sc, *S. cerevisiae*. **A)** ScYps1 (YLR120C), ScYps6 (YLR139C), CgYps1 (CAGL0M04191g), CgYps2 (CAGL0E01419g), CgYps11 (CAGL0E01881g); **B)** ScYps2 (YDR144C); **C)** ScYps3 (YLR121C); **D)** ScYps7 (YDR349C), CdYps (Cd36_18370), CpYps (CPAG_04785), CpYps (CPAG_04801), CpYps (CPAG_04802), CpYps (CPAG_03253), CpYps (CPAG_02564), CguYps (PGUG_04882), CgYps3 (CAGL0E01727g), CgYps4 (CAGL0E01749g), CgYps7 (CAGL0A02431g), CgYps9 (CAGL0E01837g), CaYps (orf19_852), CdYps (Cd36_72090); **E)** CdYps (Cd36_15430), CaYps (orf19.2082); **F)** CtYps (CTRG_01112); **G)** CpYps (CPAG_04713); **H)** CgYps8 (CAGL0E01815g), CgYps10 (CAGL0E01859g); **I)** ClYps (CLUG_00903); **J)** CtYps (CTRG_05014); **K)** CgYps5 (CAGL0E01771g), CgYps6 (CAGL0E01793g); **L)** CgYps12 (CAGL0J02288g); **M)** CaYps (orf19.6481); **N)** CaYps (orf19_853), CdYps (Cd36_18360).

detected by TRUST (Szklarczyk & Heringa, 2004) even when it is likely that the Yps and Sap superfamilies have duplicated aspartyl protease motifs.

The analysis of possible evolutive and molecular events that has given place to the presence of different numbers of *YPS* in each pathogenic *Candida* species was made to establish the COGs between Yps. Phylogenetic analysis was performed by an alignment of *YPS* homologues identified *in silico* and those of the previously characterized. The alignment was carried out using MUSCLE in SeaView 2.4 program (Galtier et al. 1996) with default alignment parameter adjustments. The phylogenetic analyses were performed in the MEGA4 program (Tamura et al. 2007) using minimum evolution computed with the Poisson correction. A similitude and identity matrix were computed with the MatGAT4.50.2 software (Campanella et al. 2003). To corroborate support for the branches on trees, bootstrap analysis (1,000 replicates) was performed. Synteny analysis was made to recognize the putative COGs (Fig. 5).

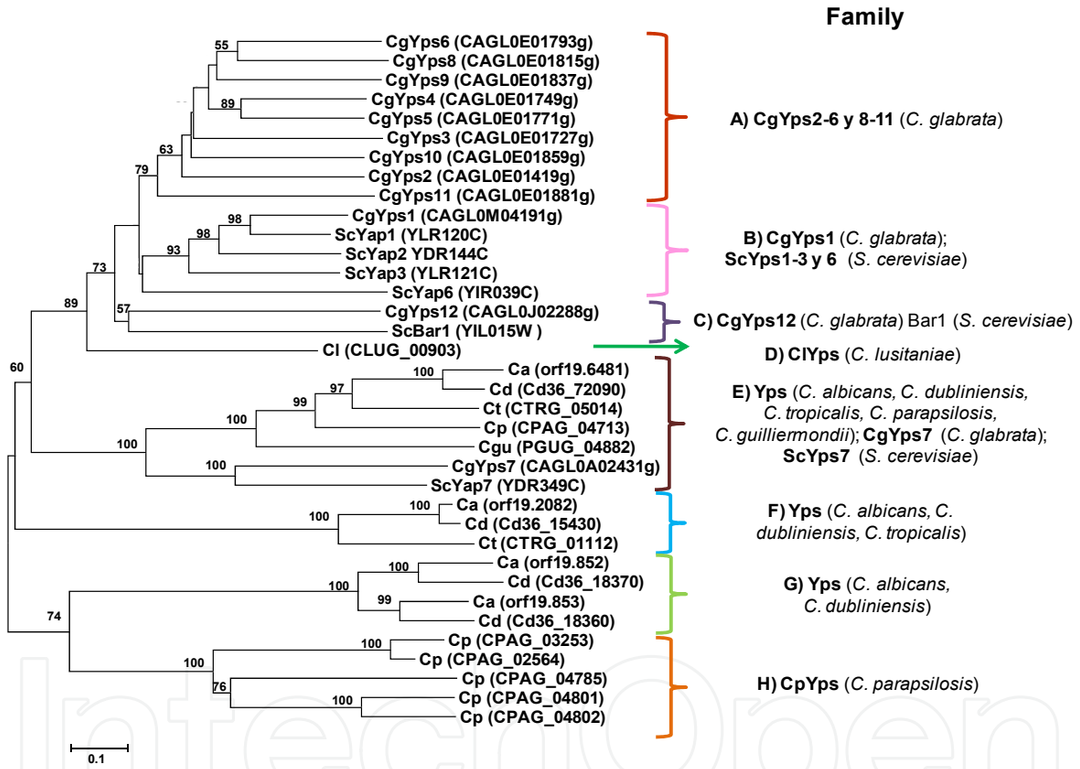

Fig. 5. Minimum evolution phylogenetic tree of GPI-anchored aspartyl proteinase (Yps) superfamily of opportunistic pathogenic *Candida* species. Ca, *C. albicans*; Cd, *C. dubliniensis*; Cg, *C. glabrata*; Cgu, *C. guilliermondii*; Cl, *C. lusitaniae*; Cp, *C. parapsilosis*; Ct, *C. tropicalis;* Sc, *S. cerevisiae*. Bootstrap values > 50% are on branches. Curly brackets and arrows indicate the Yps protein families defined by phylogenetic relationships, similitude percentage (> 50%), synteny and motif array. Yps are grouped into 8 families. Family A, CgYps2-6 and 8-11; family B, CgYps1, ScYps1-3 and ScYps6; family C, CgYps12 and ScBar1; family D, ClYps (*C. lusitaniae*); family E, CgYps7, ScYps7, CaYps (orf19.6481), CdYps (Cd36_72090), CtYps (CTRG_05014), CpYps (CPAG_04713) and CguYps (PGUG_04882); family F, CaYps (orf19.2082), CdYps (Cd36_15430) and CtYps (CTRG_01112); family G, CaYps (orf19.852), CdYps (Cd36_18370), CaYps (orf19.853) and CdYps (Cd36_18360); family H, CpYps (CPAG_03253, CPAG_02564, CPAG_04785, CPAG_04801 and CPAG_04802).

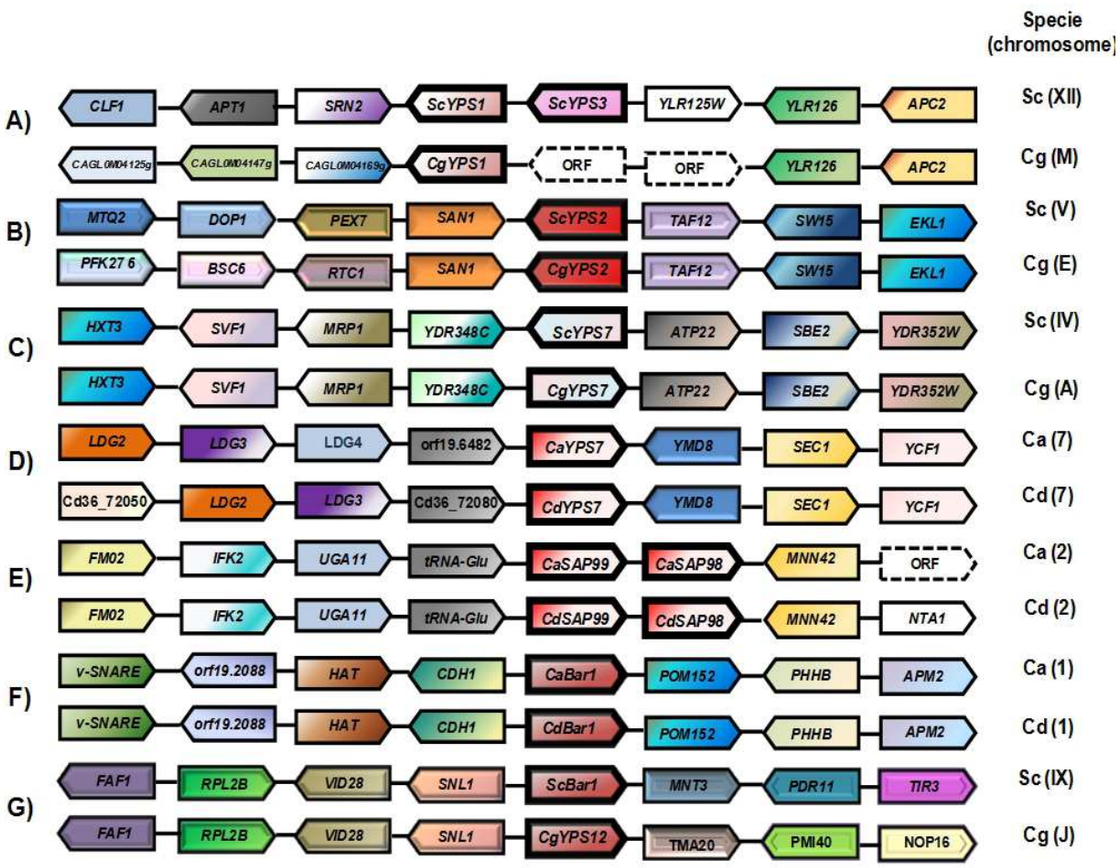

Fig. 6. Synteny of *YPS* genes of *S. cerevisiae* (Sc), *C. glabrata* (Cg), *C. albicans* (Caand *C.dubliniensis* (Cd). **A)** *ScYPS1* and *CgYPS1;* **B)** *ScYPS2* and *CgYPS2;* **C)** *ScYPS7* and *CgYPS7;* **D)** *CaYPS7* (orf19.6481) and *CdYPS7* (Cd36_72090); **E)** *CaYPS* (Sap99), *Cd* (orf19.853 and Sap98, orf19.852); **F)** *CaYPS* and *CdYPS* (Bar1); **G)** Cg*YPS* and *Sc*Bar1. *CgYPS1, CgYPS7, ScYPS1*, *ScYPS3*, Ca*YPS7*, Ca*SAP98, CaSAP99* and *BAR1* are GPI anchored aspartyl proteases; *APC2* and CAGL0M04235g, subunit of the anaphase-promoting; *APT1*, acyl-protein thioesterase; *ATP22*, mitochondrial inner membrane protein; CAGL0M04147g, similar to low affinity vacuolar membrane, is a localized monovalent cation/H+ antiporter protein; CAGL0M04169g, similar to cell wall glycoprotein involved in beta-glucan assembly; *CDH1*, cell-cycle regulated activator of the anaphase-promoting complex/cyclosome (APC/C); *CLF1*, crooked neck-like factor; *DOP1*, protein essential for viability; *EKL1*, ethanolamine kinase; *FAF1*, protein required for pre-rRNA processing and 40S ribosomal subunit assembly; *HAT*, histone acetyltransferase; *HXT3*, low affinity glucose transporter of the major facilitator superfamily; *LDG3* and *LDG4*, leucine, aspartic acid, glycine rich; *MNN42*, putative positive regulator of mannosylphosphate transferase; *MNT3*, alpha-1,3-mannosyltransferase; *MTQ2*, S-adenosylmethionine-dependent methyltransferase; *MRP1*, mitochondrial ribosomal protein of the small subunit; *NOP16*, constituent of 66S pre-ribosomal particles; *NTA1*, amidase; orf19.2088, shared subunit of DNA polymerase (II) epsilon and of ISW2/yCHRAC chromatin accessibility complex; PDR11, ATP-binding cassette transporter, *PEX7*, peroxisomal signal receptor; *PFK27*, 6-phosphofructo-2-kinase; *PHHB*, transposon mutation affects filamentous growth; *PMI40*, mannose-6-phosphate

isomerase; *POM152*, nuclear pore membrane glycoprotein; *RPL2B*, protein component of the large ribosomal subunit; *SAN1*, ubiquitin-protein-ligase; *SBE2*, protein involved in the transport of cell wall components from the Golgi to the cell surface; *SEC1*, Sm-like protein involved in docking and fusion of exocytic vesicles through binding to assembled SNARE complexes at the membrane; *SNL1*, putative protein involved in nuclear pore complex biogenesis and maintenance; *SRN2*, component of the ESCRT-I complex; *SVF1*, protein with a potential role in cell survival pathways; *SW15*, transcription factor that activates transcription of genes expressed at the M/G1 phase boundary and in G1 phase; *TAF12*, subunit (61/68 kDa) of TFIID and SAGA complexes; *TIR3*, cell wall mannoprotein of the Srp1p/Tip1p family of serine-alanine-rich proteins; *TMA20*, protein associated with ribosomes with a putative RNA binding domain; *UGA11*: gamma-aminobutyrate transaminase (4-aminobutyrate aminotransferase); *VID28*, protein involved in proteasome-dependent catabolite degradation of fructose-1,6-bisphosphatase (FBPase); *v-SNARE*, component of the vacuolar SNARE complex involved in vesicle fusion; *YCF1*, putative glutathione S-conjugate transporter; YLR126C, protein with similarity to glutamine amidotransferase proteins; *YMD8*, putative nucleotide sugar transporter; ORF, *APM2*, *BSC6*, CAGL0M04125g, Cd36_72050, Cd36_72080, *FM02*, *IFK2*, orf19.6482, *RTC1*, tRNA-Glu, *YDR352W*, YDR348C and YLR125W and ORF, unknown predicted open reading frame.

The lack of *SAP* genes and the expansion of 12 *CgYPS* genes in *C. glabrata,* and the extended family of *SAP* genes in *C. albicans* support the hypothesis that both protein superfamilies are an example of convergent evolution. Although more research is necessary to reach definite conclusions, apparently *YPS* of *C. glabrata* and *SAP* of *C. albicans* have developed some equivalent physiological functions and roles in virulence. The rest of pathogenic *Candida* species are less virulent, and, curiously, harbour less genes in their genomes than *C. albicans*. These facts lead to the supposition that *SAP* and *YPS* have evolved in an independent way for at least 700 million years. However, more *SAP* duplication events have happened in *C. albicans* (Parra et al. 2009).

Phylogenetic analyses of Yps deduced protein sequences of *Candida* spp. and *S. cerevisiae* allow for the definition of 8 Yps families, A-H (Fig. 5). In particular, CgYps1-12 proteins of *C. glabrata* were clustered in four families. Family A was constituted exclusively of nine Yps of *C. glabrata* (CgYps2-6 and CgYps8-11) encoded in chromosome E. With exception of CgYps2, all codifying genes of these proteins are organized in tandem, and possibly derived from at least eight recent duplication events that occurred exclusively in the *C. glabrata* genome. Apparently these recent duplications led to the emergence of a paralogous gene family with novel or slightly different functions. No pseudogenes were detected in *CgYPS1-11* genes, but in their deduced proteins a moderate amino acid similitude (48-53%) and identity (36-38%) were retained. Frequently, very high similitudes are maintained by concerted evolution in paralogous members of some multigene families (László, 1999). However, in *CgYPS* genes, this evolutive phenomenon is not evident. Previously, *CgYPS4* and *CgYPS11* were recognized as GPI anchored aspartyl proteases (Kaur *et al.,* 2007), but comparative studies of the regulatory region and expression of each *CgYPS* genes are necessary to clearly define the physiological role and orthology relationships of each gene. Family B was formed by a set of Yps proteins, detected exclusively in *S. cerevisiae* (ScYps2-3 and ScYps6), and a highly similar putative orthologous pair (*ScYPS1*/*CgYPS1*) (Fig. 6A). Also, the partial synteny observed between the *ScYPS2*/*CgYPS2* gene pair supports the hypothesis that those protein-coding genes are probable orthologous (Fig. 6B). Family C was

integrated by CgYps12 and ScBar1 of *S. cerevisiae*, a putative orthologous pair with low similitude synteny, but with a clear ancestor-descendant relationship (Fig. 6G). Finally, family E was formed by a representative of each *Candida* spp. Yps, CgYps7 and ScYps7. This family forms a sub tree with the same topology as those phylogenies constructed with ribosomal and other protein sequences (Diezman *et al.,* 2004). The CgYPS7 and ScYPS7 genes exhibited an extensive synteny (Fig. 6C), but no synteny with *CaYPS* (orf19.6481) and *CdYPS* (Cd36_72090) was observed (Fig. 6D). In *C. albicans* and *C. dubliniensis* genome databases these *YPS* are described as *ScYPS7* orthologous genes (Schaefer *et al*., 2007). Nevertheless, both *YPS* exhibited low similarity with *ScYPS7* (37.2-38.7%) and no-synteny. The final decision to consider family E as an orthologous family will depend on comparative analyses of functional features not yet performed.

Families C, F, G and H have not any *C. glabrata* or *S. cerevisiae* Yps representative protein. Families C and H were formed only by one ClYps gene of *C. lusitaniae* and seven CpYps genes of *C. parapsilosis*, respectively (Fig. 5). Curiously, *C. lusitaniae* is the species that harbours the fewest Cl*YPS* (n=1) and *SAP* (n=3) genes, and its isolation frequency from clinical samples ,as well as its virulence, are lower than the other *Candida* species (Abi-Said et al. 1997). This evidence supports a hypothesis of relevance of aspartyl proteases in virulence. That is, species with numerous aspartyl proteases in virulence; species with broad aspartyl proteases are more virulent than those with a limited number of these proteins.

Family F harboured *C. albicans, C. dubliniensis* and *C. tropicalis* yapsins organized congruently according to the ribosomal phylogenetic tree. The *C. albicans* CaBar1 (orf19.2082) and *C. dubliniensis* CdBar1 (Cd36_15430) gene, found in family F, has been described as orthologous to *S. cerevisiae BAR1* (Schaefer *et al.,* 2007) found in family C. In both species, *C. albicans* and *S. cerevisiae*, the protein is involved in alpha pheromone degradation and secreted to the periplasmic space of mating alpha-type cells. These proteins help cells find mating partners by cleaving and inactivating the alpha factor, which allows cells to recover from alpha-factor-induced cell cycle arrest (Mackay *et al.,* 1988). The *in silico* analysis performed in this work established that these proteins and the Bar1 from *C. dubliniensis* are extracellular, but anchored to the cell wall or cell membrane. Also, phylogenetic analysis shows that Bar1 from *C. albicans* and *C. dubliniensis* belongs to the Yps superfamily, with a similarity of 40%, and are not grouped with CgYps12 of *C. glabrata* (CgYps12 or CgBar1) and Bar1 of *S. cerevisiae*. The reason for which an aspartyl protease, that apparently is secreted, is groupedwith the yapsines superfamily could be a mistake in the cell location method because almost all software use the signal peptide, transmembranal regions, and the GPI site in the C-terminal, to predict the cell location. In *C. albicans* it has been detected that aspartyl proteases are associated with the plasmatic membrane, or to both the plasmatic membrane and cell wall. This makes the experimental corroboration of the cell location necessary. The Bar1 protein of *C. albicans* has been described as a protein with three domains: 2 aspartyl protease domains and another unidentified. Apparently, this GPI-membrane anchored domain determines that Bar proteins are not secreted, but anchored to cellular membranes, and their two actives sites are oriented to cellular membranes, and their two actives sites are oriented to the exterior to inactivate alpha pheromone, which is secreted by Mat-alpha cells. In *C. albicans*, the degradation of secreted alpha pheromone is not exclusive to Bar1. CaYPS7 (orf19.6481) of family E also encodes for this function with lesser efficiency (Schaefer *et al*., 2007). This physiological redundancy has not been demonstrated in *S. cerevisiae* ScYps7. *C. albicans* can mate under some *in vitro* and *in*

vivo conditions when alpha pheromone is degraded (Hull *et al.,* 2000; Magee & Magee, 2000) and *C. glabrata* harbours homologous genes of *S. cerevisiae* that control the mating (Srikantha *et al.,* 2003). Nevertheless, in *C. glabrata* a cell cycle has not been demonstrated, and the participation of CgYps7 of *C. glabrata* in alpha pheromone inactivation has not been demonstrated. No possible gene orthologous to possible gene orthologous to ScBar1 was detected in *C. guilliermondii*, *C. lusitaniae, C. parapsilosis, C. tropicalis, C. guilliermondii* or *C. lusitaniae*. All these yeasts have a heterothallic sex cycle (cross-mating only), but *C. parapsilosis* and *C. tropicalis* mating has never been observed (Butler *et al.,* 2009).

Family G is formed by two *C. albicans*/*C. dubliniensis* Yps protein pairs with high similitude (>88%), located in tandem in chromosome 2 and with very similar synteny. All this data is evidence from the recent speciation of both species (Fig. 6E). According to the *Candida* genome database (http://www.candidagenome.org/cgi-bin/locus.pl?locus=orf19.852) Cal orf19.852 and Cdu Cd36_18370 sequences are described as *CaSAP98* and *CdSAP98* genes, respectively, and have their best hits with *PEP4* of *S. cerevisiae* (Pra protein). *S. cerevisiae* PrA is a vacuolar protease, and clearly *C. albicans*/*C. dubliniensis* Yps are not phylogenetically grouped with PrA. In our opinion no orthology relationship among these proteins exists. Cal orf19.853 and Cdu Cd36_18360 formed a second pair, described as *CaSAP99* and *CdSAP99* genes, which had their best hits with *ScYPS3* of *S. Cerevisiae.* Similarly, it is clear that *CaSAP99* has no synteny, phylogenetic relationship, or possible common physiological role with *ScYPS3*.

3. Conclusion

Why have *C. albicans/C. dubliniensis* and *C. glabrata*/*S. cerevisiae* been suffering some genetic duplication events in their Sap and Yps superfamilies? This is something that has not been resolved, but it is clear that the decrease in virulence in null mutants, in both *CaSAP* and *CgYPS,* endorse the idea that the presence and expansion of *SAP* and *YPS* families is necessary for adaptation to the host, and therefore for survival and virulence. Also, species with broad aspartyl protease families are more virulent than those with a limited number of these proteins. *C. glabrata* belongs to a phylogenetic group with no pathogenic yeast, and its virulence attributes could be evolving independently from the CTG clade, where *C. albicans* is the main opportunistic pathogenic species. The expansion of the *CgYPS* gene superfamily of *C. glabrata* maintains a parallelism with the expansion of the *SAP* gene superfamily of *C. albicans*, and constitutes a possible example of convergent evolution. The transition from a commensally life style to a successful opportunistic pathogen could be related to gene expansion that encodes for each kind of aspartyl protease. A lot of experimental methodologies must be performed to recognize the orthologous gene families, as well as the virulence, participation and transition commensal-pathogen roles of aspartyl proteases, including Sap and Yps.

4. Acknowledgment

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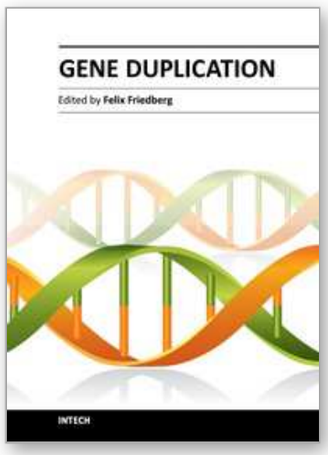

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The book Gene Duplication consists of 21 chapters divided in 3 parts: General Aspects, A Look at Some Gene Families and Examining Bundles of Genes. The importance of the study of Gene Duplication stems from the realization that the dynamic process of duplication is the "sine qua non" underlying the evolution of all living matter. Genes may be altered before or after the duplication process thereby undergoing neofunctionalization, thus creating in time new organisms which populate the Earth.

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