RESEARCH ARTICLE

Combined phylogenetic and neighbourhood analysis of the hexose transporters and glucose sensors in yeasts

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Abstract

The sugar porter family in yeasts encompasses a wide variety of transporters including the hexose transporters and glucose sensors. We analysed a total of 75 members from both groups in nine hemiascomycetous species, with complete and well-annotated genomes: *Saccharomyces cerevisiae*, *Candida glabrata*, *Zygosaccharomyces rouxii*, *Kluyveromyces thermotolerans*, *Saccharomyces kluyverii*, *Kluyveromyces lactis*, *Eremothecium gossypii*, *Debaryomyces hansenii* and *Yarrowia lipolytica*. We present a model for the evolution of the hexose transporters and glucose sensors, supported by two types of complementary evidences: phylogeny and neighbourhood analysis. Five lineages of evolution were identified and discussed according to different mechanisms of gene evolution: lineage A for *HXT1*, *HXT3*, *HXT4*, *HXT5*, *HXT6* and *HXT7*; lineage B for *HXT2* and *HXT10*; lineage C for *HXT8*; lineage D for *HXT14*; and lineage E for *SNF3* and *RGT2*.

Introduction

Saccharomyces cerevisiae natural habitat is sugar-rich fruit juices. The uptake of sugars across the plasma membrane is a decisive step in sugar metabolism and a rate-limiting step of glycolysis (Becker & Betz, 1972; Gancedo & Serrano, 1989). Since their discovery (Lewis & Bisson, 1991; Ko *et al.*, 1993; Wendell & Bisson, 1993; Theodoris *et al.*, 1994; Reifenberger *et al.*, 1995), the phylogeny of the sugar porter (SP) family of *S. cerevisiae* has been analysed by different authors (Kruckeberg, 1996; Nelissen *et al.*, 1997; Wieczorke *et al.*, 1999). More recently, 214 putative sugar porters were identified from eight hemiascomycetous yeasts *Candida glabrata*, *Kluyveromyces lactis*, *Eremothecium gossypii*, *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Candida albicans*, *Pichia stipitis* and *S. cerevisiae* (Palma *et al.*, 2007). The transported substrates were classified according to the transport classification system (Saier *et al.*, 2006) in nine clusters: hexose transporters, glucose sensors, general α-glucoside: H^+ symporter, fructose: H^+ symporter, lactose: H^+ symporter, high-affinity glucose transporters, myo-inositol: H^+ symporter, glycerol: H^+ symporter and quinate: H^+ symporter. Approximately one-third of the 214 putative sugar porters still has unknown function.

In *S. cerevisiae*, the members of the SP family span a size range of 486–884 amino acids. The largest proteins are the glucose sensors, which have a long C-terminal tail (Özcan *et al.*, 1996). The 12 putative transmembrane domains of the hexose transporters are highly conserved in contrast to the variable cytosolic amino- and carboxyl-termini (Kruckeberg, 1996).

In *S. cerevisiae*, the hexose transporters are encoded by the *HXT1* to *HXT17* and *GAL2* genes (for reviews, see Lagunas, 1993; Bisson *et al.*, 1993; Boles & Hollenberg, 1997; Leandro *et al.*, 2009) and operate through a facilitated diffusion mechanism (Jansen *et al.*, 2002; Maier *et al.*, 2002). The most important hexose transporters are Hxt1p, Hxt2p, Hxt3p, Hxt4p, Hxt6p and Hxt7p; each of these six transporters is able to support growth on glucose (Reifenberger *et al.*, 1995). They also transport fructose and mannose (Wieczorke *et al.*, 1999). Two other genes in SP family, *SNF3* and *RGT2*, act as glucose sensors (Özcan *et al.*, 1996), which do not transport glucose, but are responsible for sensing and signalling the availability of this substrate (Kruckeberg *et al.*, 1998; Özcan *et al.*, 1998). The *HXT12* is a pseudo-gene that will not be mentioned in this study (Wieczorke *et al.*, 1999).

The increasing number of yeast genome sequences provides a wealth of new information on speciation and

genome evolution. The widely used phylogenetic analysis does not *per se* show the whole history of a specific group of genes. Other evidences such as synteny (the conserved presence of neighbouring genes along a given chromosome or chromosomal segment) and conservation of functional motifs or pathways (Dujon, 2005) will provide an improved understanding of gene lineages.

The aim of this work is to outline the evolution of hexose transporters and glucose sensors considering the availability of nine complete and well-annotated genome sequences, including *S. cerevisiae*, *C. glabrata*, *K. lactis*, *E. gossypii*, *D. hansenii* and *Y. lipolytica*, and three novel yeast genomes (J.-L. Souciet *et al.*, unpublished data) provided by the Génolevures project: *Saccharomyces kluyverii*, *Zygosaccharomyces rouxii* and *Kluyveromyces thermotolerans*.

The evolution of hexose transporters and glucose sensors was inferred taking into consideration the complementary phylogeny and gene neighbourhood analyses.

Materials and methods

Genomes

The protein sequences of *S. cerevisiae* (SACE), *C. glabrata* (CAGL), *Z. rouxii* (ZYRO), *K. thermotolerans* (KLTH), *S. kluyverii* (SAKL), *K. lactis* (KLLA), *E. gossypii* (ERGO), *D. hansenii* (DEHA) and *Y. lipolytica* (YALI) were retrieved from the Génolevures database. The *Z. rouxii* and *K. thermotolerans* genomes were recently sequenced by the Génoscope, and the *S. kluyverii* genome was sequenced by the Washington University Genome Sequencing Centre. We limited our analysis to fully annotated genomes. The Génolevures consortium annotated the new genomes of ZYRO, KLTH and SAKL. The annotation of KLLA was updated by Génolevures (J.-L. Souciet *et al.*, unpublished data). The annotation of ERGO was recently updated by Gattiker *et al.* (2007).

Subset of orthologues defined by neighbourhood and similarity (SONS)

The Génolevures family GL3C002 (Nikolski & Sherman, 2007) comprised 218 members of sugar porters (Supporting Information, Table S1), within the species studied. These protein sequences were identified by identification of orthologues by neighbourhood and similarity (IONS) (M.-L. Seret *et al.*, unpublished data). The rationale of the method is that two genes of different yeast species whose translation products belong to the same family (homologues by similarity) will be members of the same SONS if they share at least one pair of neighbours that are also homologous to each other by similarity. The process is reiterated for all possible heterospecific pairwise comparisons of homologues deduced from the protein families. Homologues that do not share a pair of homologous neighbours are divided into two distinct SONS.

Within the 218 members of sugar porters considered in the present study, 54 were selected as 'hexose transporters' and 21 as 'glucose sensors' according to phylogeny and similarities to *S. cerevisiae* members.

Phylogeny

Multiple alignment of 75 selected hexose transporters and glucose sensors sequences was calculated by MUSCLE (Edgar, 2004) and processed using the PHYLIP package (Felsenstein, 2005). In a first step, SEQBOOT, a bootstrapping tool, allowed to generate 100 data sets that were analysed by PROML. In a second step, the CONSENSE program generates a consensus phylogenetic tree. We chose a 50% threshold for the consensus tree, meaning that all the generated branches have a frequency of occurrence of $> 50\%$ in the 100 generated trees. The DENDROSCOPE application (Huson *et al.*, 2007) was used to visualize the phylogenetic trees.

Results

Figure 1 shows a phylogenetic tree of the selected 75 hexose transporters or glucose sensors of the GL3C002 family. The phylogenetic clusters are defined based on the tree nodes and the bootstrap scores and labelled a to j.

By combining the phylogenetic information contained in Fig. 1 and that given by neighbourhood analysis, a model for the differentiation of hexose transporters and glucose sensors can be proposed. Five different lineages are illustrated in Fig. 2: A gave rise to the SACE transporters Hxt1p, Hxt3p, Hxt4p, Hxt5p, Hxt6p and Hxt7p; B gave rise to Hxt2p and Hxt10p; C gave rise to Hxt8p; D is the lineage of Hxt14p; and E gave rise to Snf3p and Rgt2p. Table 1 illustrates the distribution of genes in each lineage. The column labelled 'Others' lists genes that were not included in the lineages presented because there was no evidence of a relationship either by neighbourhood or phylogeny.

Lineage A: the relationship of the Hxt1p, Hxt3p, Hxt4p, Hxt5p, Hxt6p and Hxt7p transporters (cluster a, c, d and e)

The lineage A comprises 26 members and is highly represented in SACE (six members) and CAGL (seven members). Figure 3 illustrates the relationship among lineage A transporters. Neighbour genes belonging to the same family are considered homologues by similarity. The SACE *HXT3*, *HXT6* and *HXT7* genes are on chromosome D. Hxt7p is very similar to Hxt6p, as their protein sequences differ only by one amino acid out of 570. On SACE chromosome H, three adjacent copies of hexose transporters (*HXT5*, *HXT1* and *HXT4*) share identity with the triplet *HXT3*, *HXT6* and *HXT7* located on chromosome D.

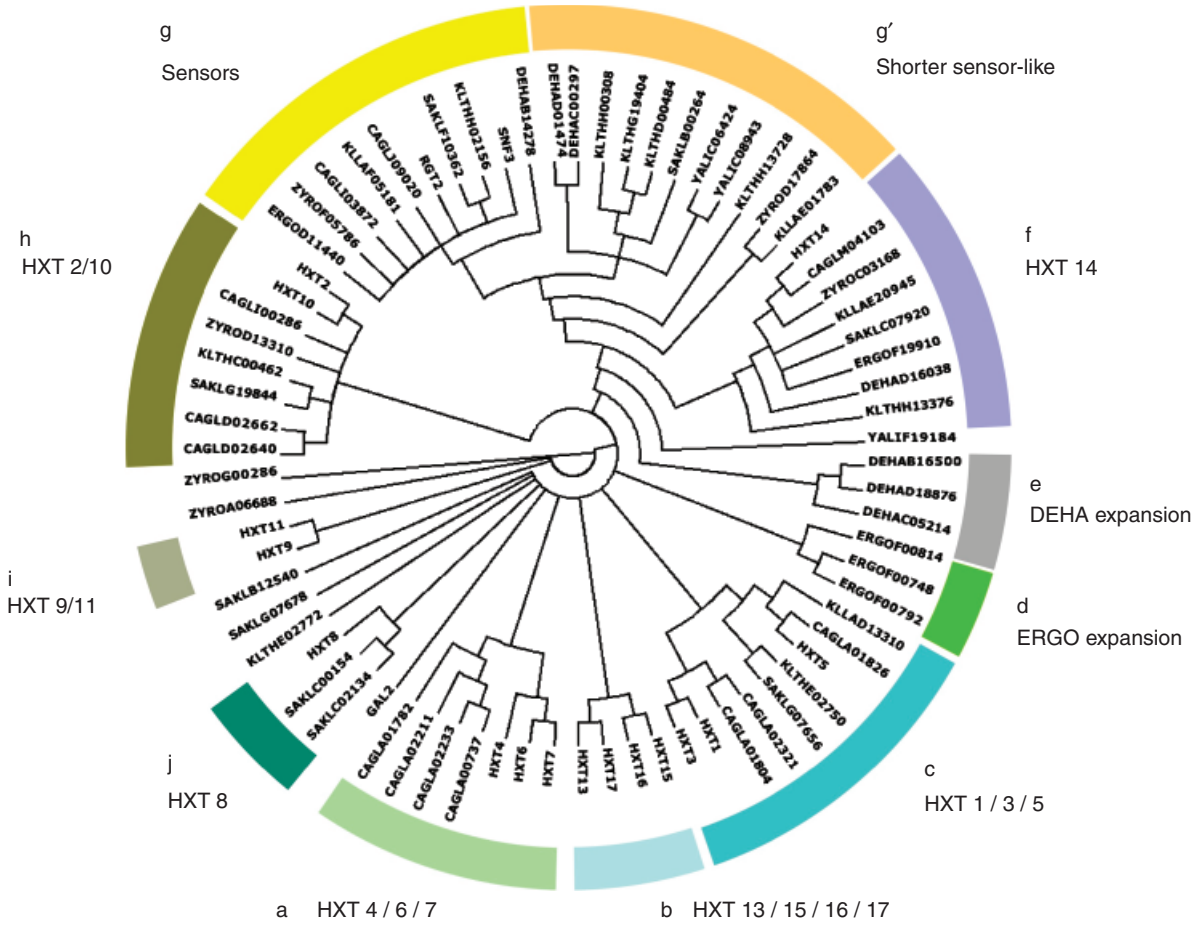

Fig. 1. A phylogenetic tree of the 75 genes based on a maximum likelihood model applied to 100 bootstrap data sets. Only nodes with > 50% support are represented.

Chromosome A from CAGL bears three members (A02321, A02233 and A02211) that share high homology with the *HXT3*, *HXT6* and *HXT7* genes, respectively. The transporters A02233 and A02211 are more different from each other than Hxt6p and Hxt7p (96% identity vs. 99% identity, respectively). Still, in CAGL chromosome A, but located 14 coding sequences apart from the A02321, A02233 and A02211 triplet, there is another set of three similar genes (A01826, A01804 and A01782). At the other end of chromosome A, the CAGL transporter A00737 is phylogenetically related to the six other CAGL members of the lineage A, sharing 79% amino acid identity with CAGL A02233. According to the SONS analysis, it is a singleton, sharing no neighbours with other members of the lineage.

In ERGO, SAKL, KLTH and ZYRO, a very significant neighbourhood (five to seven shared neighbours) connect all members of lineage A. Despite being classified in the species-specific phylogenetic cluster d, the three ERGO members share neighbours with the remaining genes in this lineage. All the members shown in Fig. 3a share at least two common families in their neighbourhood. A tandem repeat is present in SAKL and KLTH. YALI and DEHA comprise members of this lineage (amino acid identity with SACE Hxt transporters > 50%), but they are not in the same phylogenetic cluster. Figure 3b demonstrates the neighbour relationships between ZYRO, a pre-whole genome duplication (WGD) species and the post-WGD species SACE and CAGL.

Lineage B: the relationship of the Hxt2p and Hxt10p transporters (cluster h)

Lineage B consists of eight members. The SAKL subtelomeric member G19844 shares four neighbours with KLTH C00462. Other members are phylogenetically related, but cannot be confirmed by neighbourhood analysis. In CAGL and SACE, we observe once more an increase in the number of glucose transporters per species: three for CAGL and two for SACE. The ZYRO member and the CAGL I00286 share a

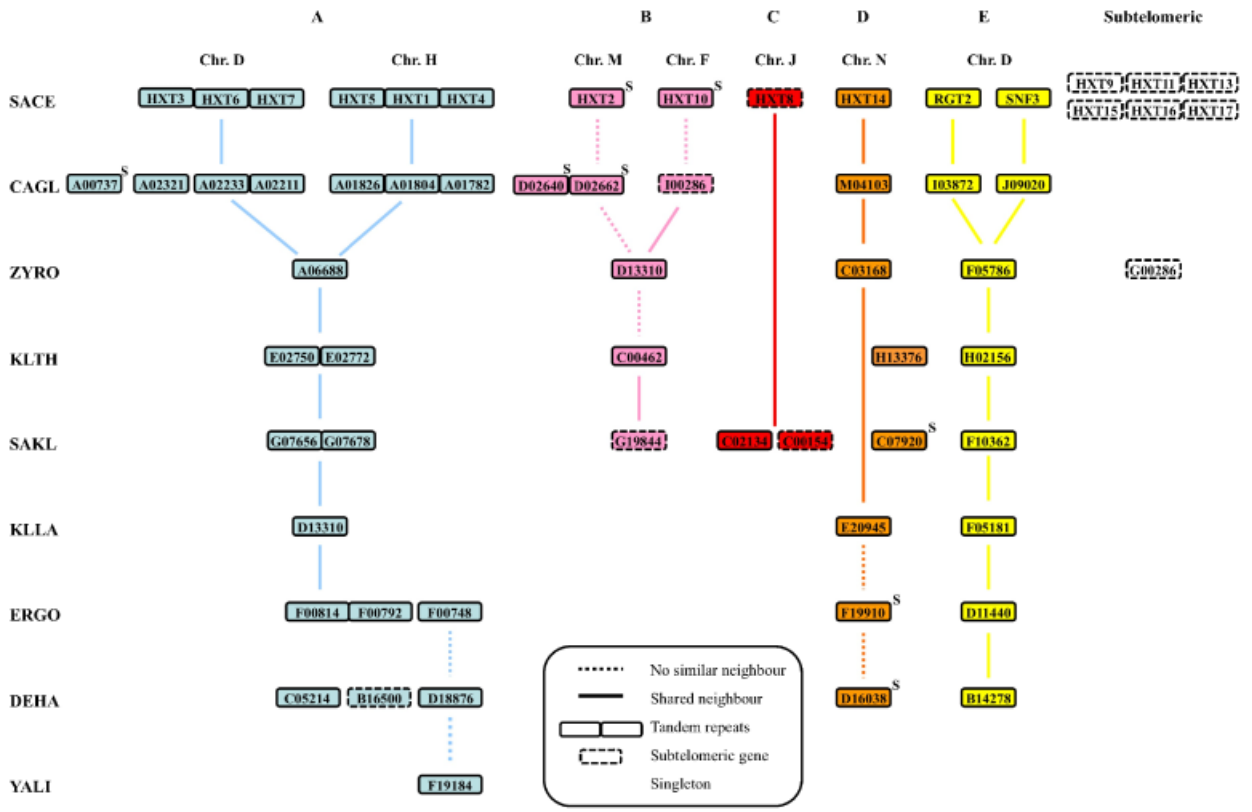

Fig. 2. The five different lineages (A to E) of hexose transporters and glucose sensors. The members shown in dashed boxes were encoded by genes located in subtelomeric regions of the chromosome. Boxes with a superscript 'S' represent genes considered as singletons by neighbourhood analysis. Full lines represent the genes that share a neighbourhood, and dashed lines illustrate relationships based on phylogeny or identity only.

Table 1. Number of genes in each lineage

	Lineage						
Species	A	B	C	D	E	Others*	Total
SACE	6	2	1	1	2	7	19
CAGL	7	3		1	2		13
ZYRO	1	1		1	1	2	6
KLTH	2	1		1	1	4	9
SAKL	2	1	2	1	1	2	9
KLLA	1			1	1	1	4
ERGO	3			1	1		5
DEHA	3			1	1	2	7
YALI	1					2	3
Total	26	8	3	8	10	20	75

*The genes in this column were not included in the lineages described in Fig. 2.

common neighbour and the latter is located in the subtelomeric region of chromosome I.

Lineage C: the relationship of the Hxt8p transporter (cluster j)

The lineage C comprises three members: SACE Hxt8p, SAKL C00154 and SAKL C02134. Both SAKL transporters share 78% and 74% amino acid identity with Hxt8p, respectively. They share both the same phylogenetic cluster and one neighbour. *HXT8* and SAKL C00154 are located in subtelomeric regions.

Lineage D: the relationship of the Hxt14p transporter (cluster f)

Lineage D consists of eight members and was established by a combination of neighbourhood and phylogeny. The

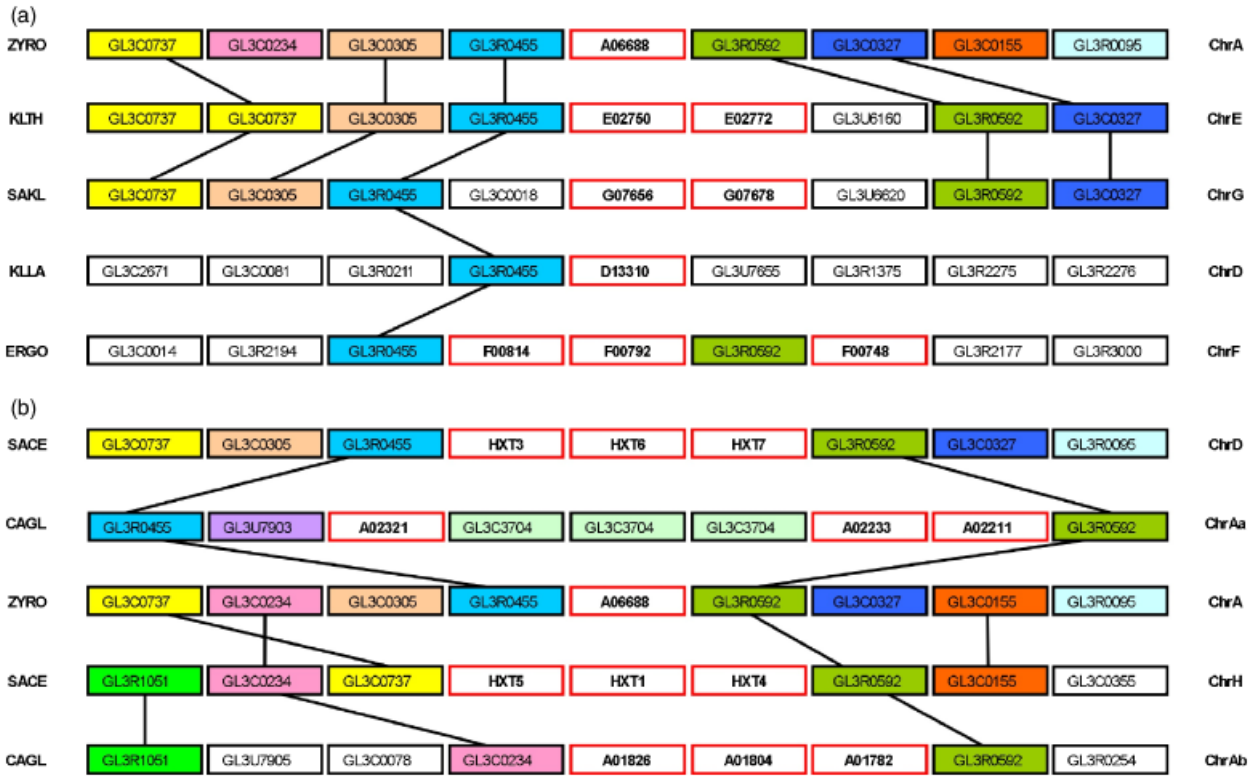

Fig. 3. Neighbourhood analysis of lineage A in (a) ERGO, KLLA, SAKL, KLTH and ZYRO and (b) ZYRO, CAGL and SACE. Each line represents a distinct chromosomal region around the hexose transporter (in red). The ZYRO chromosomal segment is repeated on both parts of the figure and connects the pre-WGD species (a) and the post-WGD species (b). Neighbours with equally coloured boxes represent members of the same family.

hexose transporters from KLLA, ZYRO, CAGL and SACE share three or more neighbours, which make this a clear lineage. The DEHA D16038, ERGO G19910 and SAKL C07920 are singletons in neighbourhood analysis but they are part of phylogenetic cluster f. They share 39%, 41% and 48% amino acid identity with SACE Hxt14p, respectively. KLTH H13376, which shares 36% amino acid identity with Hxt14p, was added to this lineage based on phylogeny only.

Lineage E: the relationship of the glucose sensors Snf3p and Rgt2p (cluster g)

Lineage E includes cluster g members that share sequence similarities with Snf3p and Rgt2p. Figures 1 and 4 show that cluster g comprises 10 members, one per species in DEHA to ZYRO, and two copies in CAGL and SACE that clearly result from WGD. No YALI member is detected in this cluster. The genes in lineage E share a consistent neighbourhood. The encoded proteins comprise from 664 to 884 amino acid residues (compared with 533–592 residues for the HXT sequences) with a 100–300 amino acid cytoplasmic C-terminus including the 25-amino acid sequence defined as the Özcan motif (Özcan *et al.*, 1998).

Although cluster g′ shares sequence similarities with this lineage, its 11 members are shorter (457–578-amino acid residues) than the glucose sensors of cluster g. Members from the g′ cluster are identified in YALI, DEHA, KLLA, SAKL, KLTH and ZYRO, but not in ERGO, CAGL and SACE. A transition between clusters g and g′ is observed in DEHA. Despite sharing neighbours with the other members of cluster g and being characterized by its long sequence of 731 amino acids, DEHA B14278 lacks the Özcan sensor signature. On the other hand, DEHA C00297 and DEHA D01474, comprising 457 and 527 amino acid residues, respectively, clearly belong to cluster g′.

Other genes

A total of 20 transporters were not included in any lineage because there were no reliable neighbourhood and phylogenetic data. The location of some genes in subtelomeres does not allow neighbourhood analysis because these are dynamic regions.

An important number (21%) of the sugar porters analysed is located in subtelomeric regions of chromosomes. As proposed by Fairhead & Dujon (2006), we considered

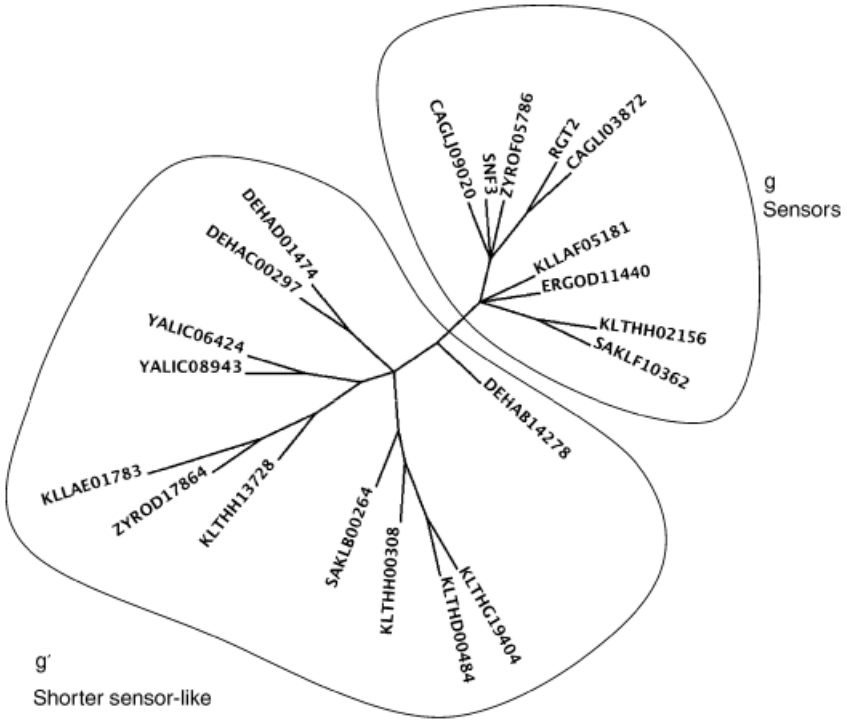

Fig. 4. Phylogeny of sensors and sensor-like proteins based on a maximum likelihood model applied to 100 bootstrap data sets. Only nodes with $>$ 50% support are represented.

subtelomeres being the last 30 kb of each chromosome. SACE possesses seven genes in subtelomeric regions: *HXT8*, *HXT9*, *HXT11*, *HXT13*, *HXT15*, *HXT16* and *HXT17* (clusters b, j and i). The *HXT15* and *HXT16* genes are located in the subtelomeric regions of chromosomes D and J, respectively. They share 99% amino acid identity and a considerable number of neighbours of the same family. The same occurs with *HXT13* and *HXT17*, which share 98% amino acid identity and are located in chromosome E and N arms, respectively. *HXT9* and *HXT11* are in chromosomes J and O, respectively, and they share 97% amino acid identity. They are also allocated in the same phylogenetic cluster and share a neighbour of the same family.

Despite sharing 76% amino acid identity, SACE Gal2p and SAKL B12540 were not considered as a lineage because there was no neighbourhood or phylogenetic evidence to justify it.

Discussion

It is thought that yeasts have separated from other organisms about one billion years ago and differentiated into *Euascomycetes*, *Hemiascomycetes* and *Archiascomycetes*. The present work considers only the 'Saccharomycetes' complex from the *Hemiascomycetes* phylum (Kurtzman & Robnett, 2003) represented by seven species whose full genome sequences offer a unique opportunity to explore eukaryotic evolution mechanisms. We present a model for the evolution of two clusters from the large SP family, the hexose transporters and glucose sensors, supported by two types of evidences: phylogeny and neighbourhood analysis.

Five lineages of evolution were identified from YALI to SACE. Each lineage can be described according to three different mechanisms: (1) conservation, gain or loss of genes during differentiation of each species, (2) WGD and (3) single-gene or segmental duplications within species.

The lineage of the Hxt1p, Hxt3p, Hxt4p, Hxt5p, Hxt6p and Hxt7p transporters involves the occurrence of the three mechanisms. We assume that the YALI putative hexose transporter F19184 represents the ancestor of the whole lineage, despite the fact that it shares no neighbours with the other studied species. Regarding the other members of the lineage, we identified a gene triplication in ERGO. Two of these three genes were lost in KLLA. This could be related to the low concentrations of hexoses in the natural lactose-rich habitat of this species (Naumova *et al.*, 2004). In SAKL and KLTH, a duplication event occurred, which is consistent with the presence of hexoses in their natural habitat (Kurtzman & Fell, 1998). The ZYRO species has lost one member. In post-WGD species, seven and six hexose transporters of the same lineage were observed in CAGL and SACE, respectively. The quasi identity of Hxt6p and Hxt7p (one amino acid residue different) may indicate that they are recently duplicated genes. In fact, Brown *et al.* (1998) reported the occurrence of multiple duplications of the high-affinity glucose transporters HXT6 and HXT7 when a population of yeast cells underwent 450 generations of glucose-limited growth. This emphasizes the potential role

of environmental pressure on the number of copies of hexose transporters. The dynamics that led to this large number of hexose transporters cannot be detailed as multiple rearrangements masks the real sequence of evolutionary events. In SACE, lineage A comprises several of the most efficient hexose transporters. As reported by Reifenberger *et al.* (1995), each of the Hxt1p, Hxt2p, Hxt3p, Hxt4p, Hxt6p and Hxt7p is able to support glucose growth on its own. Hxt2p was considered part of another lineage, together with Hxt10p. Both were considered singletons by neighbourhood analysis probably because they were translocated to another neighbourhood.

Gene loss occurred in the Hxt8p lineage as SAKL and SACE are the only species that have members of this lineage.

The 10 members of the phylogenetic cluster g (the glucose sensors) as well as the 11 members of cluster g′ (the shorter sensor-like proteins) were identified by similarity to the SACE glucose sensors Snf3p and Rgt2p. Their distribution in the studied hemiascomycetous species suggests the following scenario. The YALI members of cluster g′ comprising from 457- to 578-amino acid residues may belong to a subfamily of putative hexose transporters of undetermined specificity. In DEHA, a copy of a short g′ gene fused to a gene fragment encoding a cytoplasmic 'signal sequence' of 100–300-amino acid residues that progressively acquired the Özcan motif. The fused gene produced the 'glucose sensors' of cluster g that was conserved from DEHA to SACE. In contrast, the original 'short g′ sensor' genes present in YALI, DEHA, KLLA, KLTH, SAKL and ZYRO were lost in species such as ERGO, CAGL and SACE.

As presented in Fig. 2 by dashed-line boxes, 13 hexose transporters are located in subtelomeric regions. Seven of them are from *S. cerevisiae*. Moreover, the huge number of subtelomeric hexose transporter genes is a characteristic of *S. cerevisiae* strains, and are absent in older species such as *Saccharomyces castellii*. It was reported by Kellis *et al.* (2003) that 69% of species-specific genes in *S. cerevisiae* are located in subtelomeres. This was confirmed in a broader analysis involving other hemiascomycetous yeasts (Fabre *et al.*, 2004). Subtelomeric gene families undergo expansions and contractions in different strains or species (Fabre *et al.*, 2004; Fairhead & Dujon, 2006). The subtelomeric genes are thought to be involved in adaptation to the environment and to promote *de novo* gene creation by recombination inside gene repeats and between different chromosomal arms. Among other genes involved in sugar utilization, the *HXT13* and *HXT17*, *HXT15* and *HXT16*, *HXT11* and *HXT9* genes are impressive examples of subtelomeric amplifications that could have occurred during extensive human use of *S. cerevisiae* in brewing, baking and winemaking.

The comparison of the SACE laboratory strain S188C with the wineyard isolate RM11-1a and the clinical strain

Table 2. Hexose transporters in some species of the 'Saccharomycetes complex'

Strain Gene	*S. cerevisiae* *S288C*[*]	*S. cerevisiae* *RM11-1a*[†]	*S. cerevisiae* *YJM789*[‡]	*C. glabrata*[§]	*S. castellii*[¶]
HXT1	1	1	1	9	1
HXT2	1	1	1		1
HXT3	1	1	1		1
HXT4	1	1[‖]	1		0
HXT5	1	1	1		1
HXT6	1	0	1		1
HXT7	1	1	1		0
HXT8	1	1[‖]	0	0	0
HXT9	1	0	1	0	0
HXT10	1	1	1	1	0
HXT11	1	1	1	0	0
HXT13	1	1	1	0	0
HXT14	1	1	1	1	1
HXT15	1	1	1	0	0
HXT16	1	0	0	0	0
HXT17	1	1	1	0	0
GAL2	1	1	1	0	0
Total	17	12	15	11	6

[*]SGD Project. '*Saccharomyces* Genome Database' (2008).

[†]*Saccharomyces cerevisiae* RM11-1a Sequencing Project. Broad Institute of Harvard and MIT (http://www.broad.mit.edu).

[‡]Wei *et al.* (2007).

[§]Sherman *et al.* (2006). *Candida glabrata* possesses nine members highly similar to the first seven hexose transporter genes *HXT1-HXT7*.

[¶]Byrne & Wolfe (2005).

[‖]Protein sequence interrupted by Stop codons.

YJM789 isolated from the lungs of an AIDS patient shows some variations of the number of hexose transporter genes (Table 2). *HXT8* and *HXT16* are absent in the pathogenic strain YJM789. *HXT9*, *HXT6* and *HXT16* are absent in the RM11-1a strain and Hxt4p and Hxt8p sequences are interrupted by stop codons. In contrast, species such as *S. castellii* carries *HXT1*, *HXT2*, *HXT3*, *HXT5*, *HXT7* and *HXT14* orthologues (Byrne & Wolfe, 2005) and *C. glabrata* contains orthologues of *HXT1-7*, *HXT10* and *HXT14* only.

In conclusion, the combination of both phylogeny and neighbourhood analysis allowed us to delineate several new aspects of the evolution of the subfamily of glucose transporters and sensors in *Hemiascomycetes*. It is, however, important to note that genome dynamics sometimes masks the real sequence of events and that for some genes it is not yet possible to identify a clear evolutionary line.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Additional information about the hexose transporters and glucose sensors.

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