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RESEARCH ARTICLE

Genome-wide identification, annotation and characterization of novel thermostable cytochrome P450 monooxygenases from the thermophilic biomass-degrading fungi *Thielavia terrestris* and *Myceliophthora thermophila*

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Abstract Cytochrome P450 monooxygenases (P450s) are ubiquitous heme-thiolate proteins that have potential biotechnological application. Thermostable-P450s that can withstand hostile industrial conditions, such as high temperatures, extremes of pH and organic solvents, are needed for biotechnological usage. Here, for the first time, we report a large number of thermostable-P450s from two thermophilic biomass-degrading fungi, *Myceliophthora thermophila* and *Thielavia terrestris*. Genome-wide P450 analysis revealed the presence of 79 and 70 P450s (P450ome) in *T. terrestris* and *M. thermophila*. Authentic P450s containing both the P450 signature domains (EXXR and CXG) were classified as follows: *T. terrestris* (50 families and 56 subfamilies) and *M. thermophila* (49 families and 53 subfamilies). Bioinformatics analysis of P450omes suggested the presence of a large number of thermostable-P450s. Based on aliphatic index cut-off (>90), 14 and 11 P450s were determined to be thermostable in *T. terrestris* and *M. thermophila*. Among the thermostable P450s, six P450s from *T. terrestris* and three from *M. thermophila* had a melting temperature (Tm) of >65 °C, suggesting their hyperthermal tolerance. Analysis of the instability index of two ascomycete P450omes revealed the presence of 12 and 19 in vitro stable P450s in *T. terrestris* and *M. thermophila*. Overall, six P450s from *T. terrestris* and four from *M. thermophila* showed both thermal tolerance and in vitro stability. Thermophilic ascomycetes P450s are of potential interest from a structural, mechanistic and biotechnological point of view, as five P450s showed higher thermal tolerance and five showed higher in vitro stability compared to the well-characterized thermostable-P450s CYP175A1 (bacteria) and CYP119 (archaea).

Keywords Aliphatic index · CYP119 · CYP175A1 · Fungi · Protein melting temperature · Thermostable P450s

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Introduction

Cytochrome P450 monooxygenases (P450s) are heme-thiolate proteins distributed across the biological kingdoms (Nelson 2013). P450s perform a wide variety of reactions, such as ω-hydroxylation of hydrocarbon chains, epoxidation, deamination and dehalogenation, aromatic hydroxylation and *N*-oxidation, as well as *N*-, *O*- and *S*-dealkylation (Sono et al. 1996; Bernhardt 2006). P450s also perform

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atypical reactions including cleavage of C–C bonds (Shyadehi et al. 1996), C–C and C–O phenol coupling (Woithe et al. 2007), Baeyer–Villiger oxidation (Isin and Guengerich 2007) and rearrangement reactions (e.g. ring formation and oxidative aryl migration) (Ortiz de Montellano and Nelson 2011). Diverse catalytic reactions performed by P450s suggest that these enzymes are capable of accepting diverse substrates. The last five decades of P450 research revealed that chemical compounds belonging to different categories, such as aliphatic, aromatic, poly/polycyclic-aromatic, poly-chlorinated, and hetero-aromatic compounds, can serve as P450s substrates.

Immense catalytic activities and diverse substrate acceptance of P450s prompted the use of these enzymes as potential catalysts (Grogan 2011) for the production of fine chemicals (Guengerich 2002) pharmaceutical compounds (Ingelman-Sundberg 2004; Guengerich 2006), antibiotics, fragrances and detoxification of carcinogenic and/or mutagenic compounds (Urlacher and Eiben 2006; Urlacher and Girhard 2011). Biotechnological utilization of enzymes on an industrial scale requires robust enzymes that can withstand industrial reaction conditions, including thermostability (Niehaus et al. 1999; Zeikus et al. 1998). However, the thermal and chemical instability of P450 enzymes hinders their practical applications (O'Reilly et al. 2011). Considering the fact that P450s are weak and less stable enzymes, research has focused on identifying thermostable P450s (Nishida and Ortiz de Montellano 2005; Li et al. 2007). Thermostable P450s can serve as a potential biocatalyst for practical industrial applications, such as the synthesis of important organic intermediates. Thermal stability also increases the stability of the protein in hostile environments, such as extremes of pH and organic solvents. Thermostable P450s furthermore give an advantage to setup reaction in a two-liquid phase system where P450 substrates (mostly organic in nature) can react with P450s in the organic phase and products (hydrophilic in nature) can enter into the water phase. This method facilitates the generation and purification of the valuable P450 products in bulk. Successful use of P450s in a two-liquid phase system has been reported (Schula et al. 2005).

To date, only a few thermostable P450s have been described in the literature and most of them are of archaeal origin. The first identified and the best studied thermostable P450 CYP119 was isolated from the acidothermophilic archaeon *Sulfolobus sulfataricus* (Wright et al. 1996; Koo et al. 2000, 2002). The best characterized thermostable P450 of bacterial origin is CYP175A1 from *Thermus thermophilus* (Blasco et al. 2004). In addition to the natural thermostable P450s, a large number of thermostable P450s with new catalytic activities were created using the well-characterized *Bacillus megaterium* P450 CYP102A1 (Li et al. 2007).

No thermostable P450s from other biological kingdoms have been reported. In this paper we report thermostable P450s from the large and diverse lower eukaryotic organisms containing kingdom, fungi. In this study we identified a large number of thermostable P450s of biotechnological potential from the two thermophilic biomass-degrading ascomycetes, *M. thermophila* (*Sporotrichum thermophile*) and *T. terrestris* (Berka et al. 2011).

Materials and methods

Fungal genome data mining for P450s

A search was conducted for publicly available genomes [JGI Mycocosm (Grigoriev et al. 2012)] of the thermophilic biomass-degrading fungi *M. thermophila* (*S. thermophile*) v2.0 (http://genome.jgi.doe.gov/Spoth2/Spoth2.home.html) and *T. terrestris* v2.0 (http://genome.jgi-psf.org/Thite2/Thite2.home.html) (Berka et al. 2011), using the term "P450". All the hit protein sequences were downloaded and subjected to ClustalW analysis (multiple and pairwise alignment) using Molecular Evolutionary Genetics Analysis (MEGA 5.0.5) (Tamura et al. 2011). The aligned protein sequences were searched for the presence of the P450 signature motifs, namely the oxygen-binding motif (FXXGXXXCXG) and the heme-binding motif (EXXR). Sequences that showed both conserved P450 signature motifs were retained and the remaining sequences were subjected to BLAST analysis (blastp at NCBI). In BLAST analysis, the sequences that showed homology to P450s were retained and the remaining sequences that showed homology to other protein families were excluded from the analysis. The sequences that showed both P450 conserved signature motifs were regarded as "authentic P450s" and the remaining sequences that showed one of the two conserved domains or showed homology to P450 proteins were grouped as "pseudo-P450s". Authentic P450s were selected for further analysis.

Annotation and phylogenetic analysis of P450s

Annotation and phylogenetic analysis of P450s were performed in the same way as described by Dr Syed in his recent publication (Syed et al. 2013a). For each of the authentic P450s a homologous P450 was identified by performing BLAST analysis using "against all named P450s" criteria at Cytochrome P450 Homepage (http://drnelson.uthsc.edu/P450seqs.dbs.html) (Nelson 2009). Based on the BLAST analysis, a table showing homolog P450 and percentage homology to each of the authentic P450s was constructed (data not shown). Each authentic P450 was assigned a family and subfamily following the

standard P450 nomenclature rule, i.e. >40 % sequence identity for assigning a family and >55 % for a subfamily (Nelson 2004). Any P450 that showed less than 55 % homology was assigned to a new subfamily (NS). Furthermore, the assigned family and subfamily naming for each of the authentic P450s was classified based on their phylogenetic placement (Nelson 2009), i.e. P450s belonging to the same family and subfamily should group together in the phylogenetic tree.

The minimum evolution method (Rzhetsky and Nei 1992) chooses the smallest value of the sum of all branches as an estimate of the tree, resulting in pairing of the true neighbor taxons (e.g. proteins) reflecting the true evolutionary aspect. This method has also been widely used in P450 research for phylogenetic analysis of P450s (Chen et al. 2012). Considering its accuracy and wide use in P450 research, in this study we used the minimum evolution method for phylogenetic analysis of P450s. Minimum evolution method based phylogenetic analysis of P450s was carried out using Molecular Evolutionary Genetics Analysis (MEGA 5.05) software (Tamura et al. 2011). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. The minimum evolution tree was searched using the close-neighbor-interchange algorithm (Nei and Kumar 2000) at a search level of 1. The neighbor-joining algorithm (Saitou and Nei 1987) was used to generate the initial tree.

Synteny analysis of P450s in thermophilic genomes

The physical localization of P450 genes on the chromosomes was carried out as described elsewhere (Nazir et al. 2010). Chromosomes and their sequences were downloaded from the respective thermophilic fungi databases at JGI. The position of P450s was marked on each chromosome and presented as a synteny figure (Figs. 2, 3). Considering the position of each authentic P450 gene on the chromosome, a synteny picture was generated (Nazir et al. 2010). *Thielavia terrestris* has six chromosomes (Fig. 2) whereas *M. thermophila* has seven (Fig. 3) (Berka et al. 2011).

Ascomycete species P450s for comparative analysis

For comparative P450 analysis, annotated P450 sequences from 14 ascomycete species, *Mycosphaerella fijiensis, Uncinocarpus reesii*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Aspergillus clavatus*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Neurospora crassa*, *Neurospora discrete*, *Fusarium graminearum* and *Fusarium oxysporum,* were downloaded from the publicly available P450 database, Cytochrome P450 Homepage (http://drnelson.uthsc.edu/P450seqs.dbs.html) (Nelson 2009). Family-level comparative analysis was carried out to check the presence of common and unique P450s in thermophilic biomass-degrading ascomycetes compared to the 14 mesophilic ascomycetes. Family-level comparison was limited to the families identified in *T. terrestris* and *M. thermophila,* considering the ascomycetes P450omes are highly diverse and comprise a large number of P450 families (Nelson 2011).

Measuring P450s thermostablity

The aliphatic index (Ikai 1980) is taken as an indicator of the thermostability of P450 proteins. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). The index is regarded as a positive factor for the increase of thermostability of globular proteins (Ikai 1980). The aliphatic index of P450 proteins was calculated using the ProtParam programme (Gasteiger et al. 2005) available at ExPASy Bioinformatics Resource Portal (http://www.expasy.org/). Each P450 sequence is analyzed individually with ProtParam and P450s showing an aliphatic index of more than 90 °C is selected as thermostable P450s. For comparative analysis of thermostablity, homologous P450 proteins from mesophilic ascomycete species were used. Two thermophilic P450 proteins, CYP119 (archaeon) and CYP175A1 (bacterial), are the only P450s that are well-characterized for their biochemical and biophysical properties with respect to thermostablity (McLean et al. 1998; Park et al. 2002; Yano et al. 2000, 2003; Blasco et al. 2004). For this reason, in the present study, these P450 proteins were used as reference protein for comparison of thermostable properties.

Measuring P450s melting temperature

Protein melting temperature (Tm) is the temperature at which 50 % of the protein is unfolded. Tm is an inherent property of a protein towards thermal tolerance. P450 proteins' Tm is calculated directly using protein sequences by means of the bioinformatics program Tm predictor available at http://tm.life.nthu.edu.tw/ (Ku et al. 2009).

Assessing the in vitro stability of P450 proteins

The instability index (II) is used as an indication of the in vitro stability of P450s. The instability index is a measure of proteins, used to determine whether they will be stable in a test tube (Guruprasad et al. 1990). If the index is less than 40, the protein is regarded as stable in the test tube and if it is greater than 40, it is regarded as not stable. The

instability index of P450 proteins was calculated using the ProtParam program (Gasteiger et al. 2005) available at ExPASy Bioinformatics Resource Portal (http://www.expasy.org/). The instability index of functionally characterized thermal P450s CYP119 (McLean et al. 1998) and CYP175A1 (Blasco et al. 2004) is used as a reference.

Results and discussion

Fungi represent large and diverse lower eukaryotes. Recent genome sequencing of fungal species revealed the presence of a large number of P450s in their genomes, with some exceptions (Moktali et al. 2012; Nelson 2011). Functional analysis of fungal P450s showed the presence of P450s displaying extraordinary catalytic activities (Syed et al. 2013b) and recently a fungal P450 has been engineered to oxidize carcinogenic and/or mutagenic compounds (Syed et al. 2013c). However, to date, P450s with a thermostable nature have not been reported from this kingdom. Genome sequencing of thermophilic biomass-degrading ascomycete species *T. terrestris* and *M. thermophila* revealed the presence of thermostable enzymes in their genomes (Berka et al. 2011). In this study we aimed to identify thermostable P450s in the two biomass-degrading ascomycetes *T. terrestris* and *M. thermophila.*

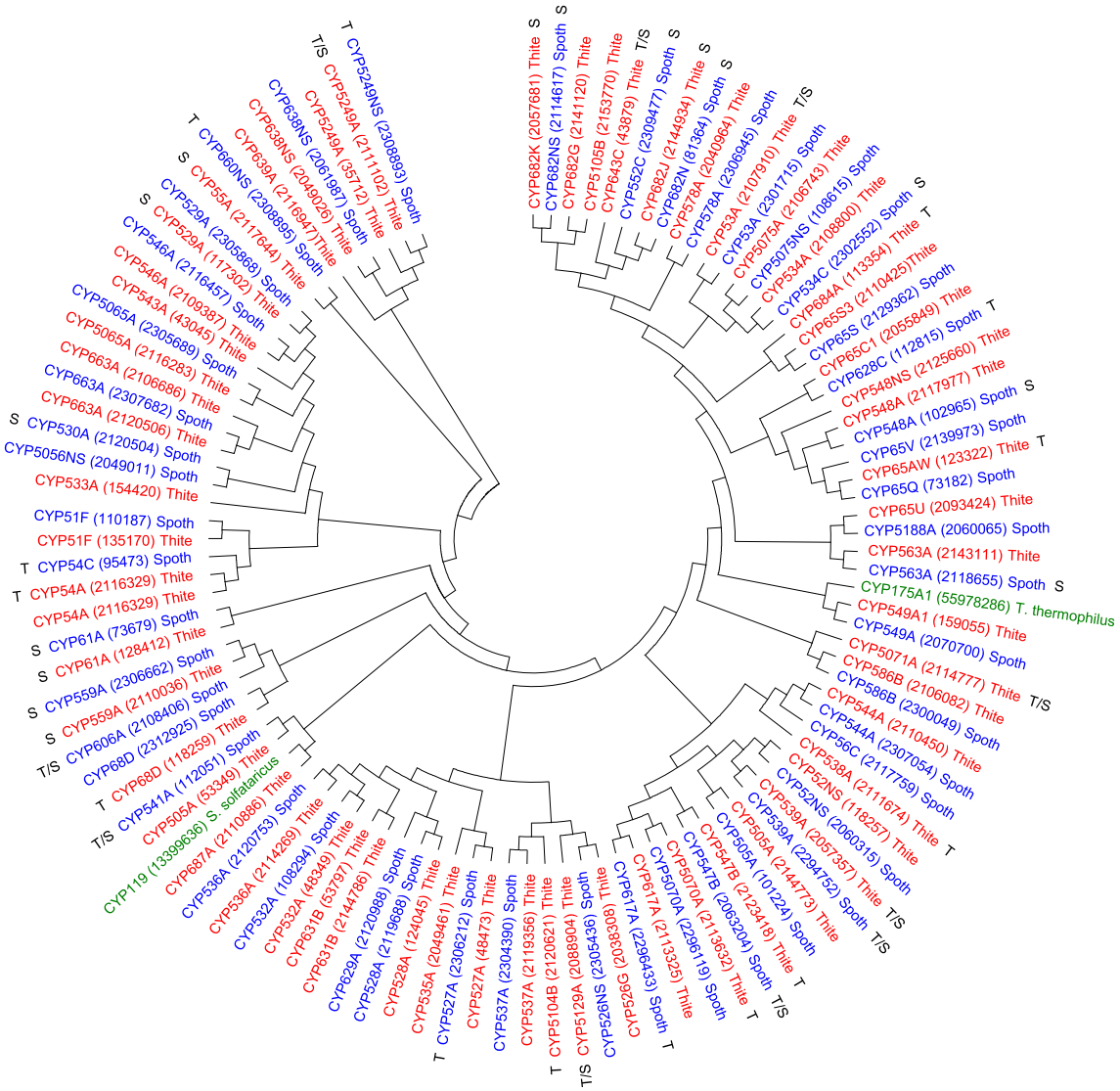

Fig. 1 Phylogenetic analysis of the P450ome of *T. terrestris* (Thite) and *M. thermophila* (Spoth). In total 108 P450 sequences from two thermophilic ascomycetes were included in the tree. Thermostable P450s CYP119 and CYP175A1 were also included for evolutionary analysis. A minimal evolution tree was constructed using the close-neighbor-interchange algorithm in MEGA (version 5.05). For ease of visual identity, P450s along with their protein IDs (parenthesis) were presented in red (*T. terrestris* P450s) and blue (*M. thermophila* P450s). Symbols next to P450s, *S* and *T* indicate stable (in vitro in a test tube) P450 and thermostable P450. (Color figure online)

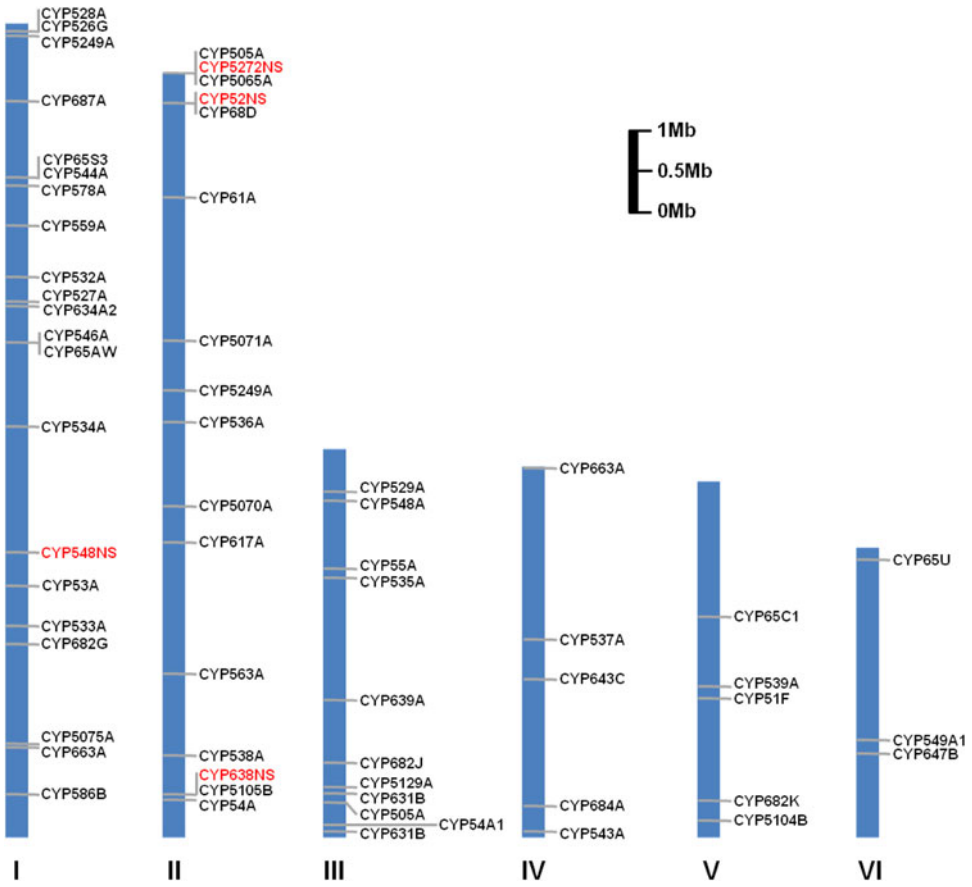

Fig. 2 Chromosomal localization of P450s in *T. terrestris*. New P450 subfamilies identified in this fungus are shown in red font. (Color figure online)

Thielavia terrestris P450ome

Genome-wide search using the term "P450" with gene ontology selection criteria yielded 96 hits. Among the 96 hits, 61 protein sequences showed both P450 signature motifs and were regarded as authentic P450s (Fig. 1; Supplementary Table 1). Eighteen hit sequences showed one of the P450 signature domains or high homology to P450s, hence they were grouped under pseudo-P450s (Supplementary Table 1). The remaining 17 sequences showed homology to non-P450 proteins, hence these were considered as non-P450s. A comprehensive list of *T. terrestris* P450s containing the CYP name, protein ID and location is presented in Supplementary Table 1.

Based on the international P450 nomenclature criteria (Nelson 2004), the authentic 61 P450s were grouped into 50 families and 56 subfamilies (Fig. 1; Supplementary Table 1). One P450 protein (ID: 53349) showed high homology to CYP505 family proteins known as fusion P450s (P450-CPR) (Nakayama et al. 1996). Interestingly, JGI automated annotation did not yield any redox (CPR) domain for this protein. We successfully predicted the redox domain and grouped this protein under the CYP505A1 family. Furthermore, phylogenetic analysis of named P450 sequences revealed family-specific grouping of P450s (Fig. 1), suggesting correct assignment of the family and subfamily to the P450 sequences. Among the 56 subfamilies, *T. terrestris* P450ome showed three new subfamilies. New subfamilies were found in P450 families CYP52, CYP548, CYP638 and CYP5272 (Supplementary Table 1).

Synteny analysis of P450s suggested that the P450 genes are well-distributed among the six chromosomes (Fig. 2). Twenty-one P450s were localized on chromosome I. The four new subfamilies are located on chromosome II (Fig. 2).

Myceliophthora thermophila P450ome

The *M. thermophila* genome showed 88 hits for the term "P450". Among these 88 hits, 53 sequences were regarded as authentic P450s (Fig. 1; Supplementary Table 2), 17 were pseudo-P450s (Supplementary Table 2) and 18 were non-P450 sequences. According to the international P450 nomenclature criteria, 53 authentic P450s were grouped into 49 families and 53 subfamilies (Fig. 1; Supplementary Table 2). A comprehensive list of *M. thermophila* P450s containing the CYP name, protein ID and location is presented in Supplementary Table 2. *M. thermophila* P450ome showed the presence of eight new subfamilies in the P450

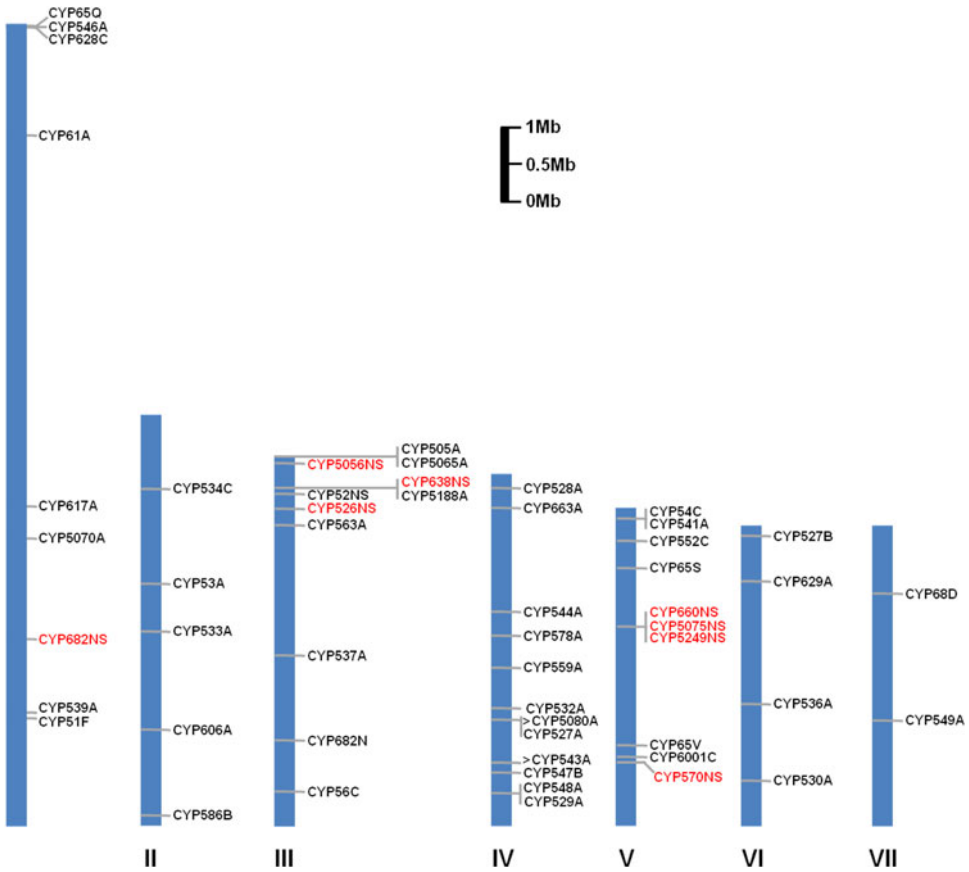

Fig. 3 Chromosomal localization of P450s in *M. thermophila.* New P450 subfamilies identified in this fungus are shown in red font. (Color figure online)

families, CYP526, CYP570, CYP638, CYP660, CYP682, CYP5056, CYP5075 and CYP5249 (Supplementary Table 2). Similar to *T. terrestris*, *M. thermophila* P450 genes are well-distributed among seven chromosomes (Fig. 3). Most of the eight new subfamilies are located on chromosome III and V (Fig. 3). Genome-wide distribution of P450s is considered a characteristic of ascomycetes, as these fungi display fewer P450 gene duplications and high diversity compared to basidiomycetes (Nelson 2011).

Comparative P450ome analysis

P450ome comparison between two thermophilic biomass degraders revealed the presence of almost the same number of P450s, 79 in *T. terrestris* and 70 in *M. thermophila*. Family-level comparison of P450omes revealed the presence of 36 common P450 families between two thermophile ascomycetes (Fig. 4). The presence of such a large number of P450 families common in both strains is understandable, considering that the thermophilic ascomycetes belong to the same family, i.e. chaetomiaceae. A total of 13 and 14 unique P450 families were found in *M. thermophila* and *T. terrestris* P450omes (Fig. 4). A comprehensive comparison of *T. terrestris* and *M. thermophila* P450omes with 14 mesophilic ascomycete species P450omes (Table 1) revealed that thermophilic ascomycete species P450ome is smaller than the *Fusarium* species, *Aspergillus* species and *M. fijiensis*. However, *T. terrestris* and *M. thermophila* P450omes are largest when compared to the P450omes from the *Neurospora* species (*N. crassa* and *N. discrete*) and *U. reesii*, *H. capsulatum*, and *Coccidioides immitis* (Table 1). Family-level P450ome comparison revealed the presence of three unique P450 families CYP563, CYP5071 and CYP5129, in two thermophilic ascomycetes, compared to 14 mesophilic ascomycetes. Interestingly, P450ome of two thermophilic ascomycetes showed diversity in terms of P450 families. *T. terrestris* and *M. thermophila* P450omes showed P450 families that are widespread across the 14 mesophilic ascomycetes (Table 1). For example, P450 families CYP634 and CYP638 found in *Fusarium* species are present in the two thermophile ascomycetes. Apart from the CYP51 and CYP61 P450 families that are well-conserved across fungi, we identified three more families (CYP53, CYP539 and CYP548) conserved across the 16 ascomycetes (Table 1).

Thielavia terrestris and *Myceliophthora thermophila* genomes as a reservoir for thermostable P450s

Genome sequencing analysis of the two thermophilic biomass-degrading ascomycetes *T. terrestris* and *M. thermophila* revealed the presence of a large number of thermostable enzymes (Berka et al. 2011). However, in this

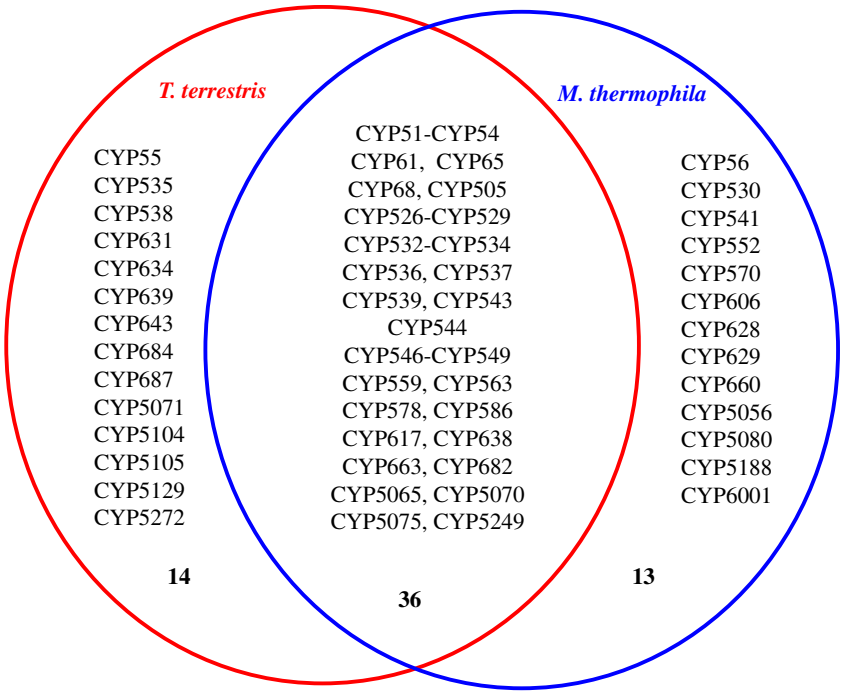

Fig. 4 Family-level comparative analysis of the P450omes of *T. terrestris* and *M. thermophila*. Numbers in boldface indicate the number of P450 families

study the cytochrome P450 enzyme superfamily is completely disregarded. Considering the thermophilic nature and identification of thermostable enzymes (Berka et al. 2011), one can expect the presence of thermostbale P450s in the two thermophiles. In this study, we investigated the thermophilic ascomycetes P450omes for thermostable P450s. We followed two criteria to select thermostable P450s, i.e. aliphatic index and Tm.

Analysis of the aliphatic index for two thermophilic ascomycete P450omes revealed the presence of a large number of thermostable P450s (Table 2). Based on the aliphatic index cut-off (>90) we found 14 and 11 P450s as thermostable P450s in *T. terrestris* and *M. thermophila* (Table 2). All selected thermostable P450s showed a higher aliphatic index compared to the P450s from mesophilic ascomycetes (Table 2), suggesting that the identified P450s are thermostable. Furthermore, to authenticate the identified thermostable P450s, the aliphatic index of the ascomycete P450s was compared with thermostable P450s from bacteria (CYP175A1) and archaea (CYP119). Three P450s, CYP5129A1 (100.43), CYP5071A (96.83) and CYP547 (102.34) from *T. terrestris,* had a higher aliphatic index compared to CYP119 (aliphatic index: 96.63). *M. thermophila* P450ome featured four P450s, CYP547B (101.3), CYP606A (97.49), CYP54C (98.56) and CYP61 (96.74), with a higher aliphatic index compared to CYP119. In comparison to CYP175A1's aliphatic index (97.20), two and three P450s from *T. terrestris* and *M. thermophila* had a higher aliphatic index (Table 2). This is the first report on the identification of thermostable P450s enzymes from fungi with an aliphatic index higher than the well-characterized thermostable P450s CYP175A1 and CYP119.

Tm is an inherent property of a protein and it indicates the thermal tolerance of the protein. A higher Tm improves thermal tolerance. According to the measurement (Ku et al. 2009), a Tm value <55 °C indicates non-thermostable protein, a Tm value between 55 and 65 °C indicates thermostable protein and a Tm value >65 °C indicates hyper-thermostable protein (Ku et al. 2009). An analysis of Tm for the identified thermostable P450s of the two ascomycetes showed that all of the P450s (except CYP538A1 from *T. terrestris*) have a Tm value in the range of thermostable protein (55–65 °C) or hyper-thermostable protein (>65 °C) (Table 2). In order to authenticate the Tm value, we also measured the Tm for CYP119 and CYP175A1. As shown in Table 2, CYP119 and CYP175A1 showed a Tm between 55 and 65 °C and above 65 °C. This indicates that the software program prediction of Tm value is accurate. Based on a Tm of above 65 °C (the same observed for CYP175A1), we conclude that six thermostable P450s from *T. terrestris* and three thermostable P450s from *M. thermophila* are hyper-thermostable P450s (Table 2).

Thielavia terrestris and *Myceliophthora thermophila* genomes are reservoirs for in vitro stable P450s

In order to identify stable P450s, two ascomycetes P450omes were subjected to instability index measurement (Guruprasad et al. 1990). The instability index is a direct measure of protein stability under in vitro conditions, such as in a test tube. It is based on the dipeptide composition of

Table 1 Comparative analysis of the P450omes of thermophilic and mesophilic ascomycetes

P450 family	Thermophilic pezizomycetes		Mesophilic pezizomycetes													
	TT	MT	MF	UR	HC	CI	AC	AN	AF	AO	AT	AFu	NC	ND	FG	FO
CYP51	1	1	1	2	2	1	2	2	4	3	3	2	1	1	3	3
CYP52	1	1	1	1			2	5	4	5	4	3				
CYP53	1	1	1	1		1	1	1	1	2	1	1	1	1	3	3
CYP54	2	1											1	1	1	1
CYP55	1			1	1			1	1		1		1	1	1	4
CYP56		1		2		1	1		1	1						
CYP61	1	1	1	1	1	1	2	2	2	2	3	1		1	2	2
CYP65	4	3	2		7	1	5	14	9	10	8	3	2	2	4	2
CYP68	1	1			2		1	3	1	2	3	1	1	1	5	2
CYP505	2	1	3				2	3	4	3	2	1	1	1	2	4
CYP526	1	1									1		1	1	2	1
CYP527	1	2						1					2	2	1	3
CYP528	1	1	1										1	2	1	
CYP529	1	1											1	1		
CYP530		1		1		1					1	1	1	1	1	1
CYP532	1	1	1					2	1	1	1		1	1	4	3
CYP533	1	1											1	1		
CYP534	1	1	1										1	1	1	
CYP535	1								1	1			1	1		
CYP536	1	1	1										1	1		
CYP537	1	1							1	1	1		1		1	2
CYP538	1												1	1		
CYP539	1	1	1	1	2	1	2	1	1		3	2	1	1	2	1
CYP541		1					1	1	1	1	1	1	1	1		
CYP543	1	1	1										1	1		
CYP544	1	1											1	1	1	1
CYP546	1	1											1	1	1	
CYP547	1	1	1					1	1	1	2	1	1	1	1	1
CYP548	2	1	3	2	1	2	4	6	2	3	3	1	1	1	2	6
CYP549	1	1											1	1		
CYP552		1							1		1		1	1	3	3
CYP559	1	1	2										1	1		1
CYP563	1	1														
CYP570		1	2	1		1									3	5
CYP578	1	1	3	2	1	2	2	3	3	2	2	2				2
CYP586	1	1			1			2			1	1				
CYP606		1					1				1				1	1
CYP617	1	1	1	1			5	3	1	1	1	3			3	3
CYP628		1			1			1	1	1	1				1	4
CYP629		1			1										1	1
CYP631	2								2	1					1	
CYP634	1														1	1
CYP638	1	1			1										1	1
CYP639	1														1	1
CYP643	1							1	1						4	1
CYP660		1				1	1	1	2	2	1	1				
CYP663	2	1	1				1	1	1		1					

Table 1 continued

P450 family	Thermophilic pezizomycetes		Mesophilic pezizomycetes													
	TT	MT	MF	UR	HC	CI	AC	AN	AF	AO	AT	AFu	NC	ND	FG	FO
CYP682	3	2	1	4	1	6	2	2	3	2	2	1				1
CYP684	1		1					2	1	1	3					1
CYP687	1								1							
CYP5056		1														
CYP5065	1	1														1
CYP5070	1	1							1	1						
CYP5071	1															
CYP5075	1	1						1	2	2						
CYP5080		1														
CYP5104	1					1		2			1					
CYP5105	1							1	1							
CYP5129	1															
CYP5188		1	1													
CYP5249	2	1			1											
CYP5272	1						1							1		
CYP6001		1	1		1	1	2	2	3		3			1		1
Total no. of P450s in the genome	61	53	89	38	47	40	91	154	162	142	124	74	41	43	109	140

P450omes of the thermophilic ascomycetes *T. terrestris* (TT) and *M. thermophila* (MT) were compared with P450omes of the 14 mesophilic ascomycete species, *Mycosphaerella fijiensis* (MF), *Uncinocarpus reesii* (UR), *Histoplasma capsulatum* (HC), *Coccidioides immitis* (CI), *Aspergillus clavatus* (AC), *Aspergillus niger* (AN), *Aspergillus flavus* (AF), *Aspergillus oryzae* (AO), *Aspergillus terreus* (AT), *Aspergillus fumigatus* (AFu), *Neurospora crassa* (NC), *Neurospora discrete* (ND), *Fusarium graminearum* (FG) and *Fusarium oxysporum* (FO). P450s belong to CYP6001 family was found in different strains of the same species. Hence, as a representative, the CYP6001 count is included in the species. Considering the presence of a large number of P450 families in ascomycetes P450omes (Nelson 2011) the comparison is limited to P450 families present in thermophilic ascomycetes. For comparative purposes the total number of P450s present in each species is also shown at the bottom of the table

a protein. An instability index of less than 40 indicates that a protein is stable and higher than 40 indicates that the protein is unstable in a test tube. Analysis of two ascomycete P450omes revealed the presence of a large number of stable P450s (Table 3). Twelve P450s from *T. terrestris* and 19 P450s from *M. thermophila* had an instability index of less than 40, suggesting their high stability in a test tube (Table 3). The software predictions were authenticated by measuring the instability index of CYP119 and CYP175A1 (Table 2). Both P450s had an instability index lower than 40. This suggests that the software predictions are accurate and the stable P450s that were identified are possibly true stable proteins. CYP643C from *T. terrestris* had the lowest instability index (31.62) compared to the stable P450s identified in two ascomycetes and CYP119 and CYP175A1, suggesting that this P450 is highly stable.

Overall, considering the thermostability and instability index, a total of 10 P450s, six P450s from *T. terrestris* and four from *M. thermophila* (Fig. 1) are biotechnologically valuable, as they showed both thermal and in vitro stability. Furthermore, five P450s showed highest thermal tolerance (Table 2) and five showed the highest in vitro stability (Table 3) in comparison to the well-characterized CYP119 and CYP175A1, suggesting these P450s are of potential interest from a structural, mechanistic and biotechnological point of view.

Functional analysis of thermophilic ascomycete P450s

Based on the homologous P450s in other fungi, the functional data of some of the thermophilic ascomycetes P450s can be predicted. CYP51 and CYP61 perform lanosterol-14α-demethylation (Lepesheva and Waterman 2004) and C-22 sterol desaturase activity (Kelly et al. 1995) in cell wall ergosterol biosynthesis. CYP52 oxidizes *n*-alkanes and fatty acids (Van Bogaert et al. 2011) and CYP53 is involved in xenobiotic compound benzoate hydroxylation (Faber et al. 2001). CYP55 is a nitric oxide reductase that performs the denitrification step in the global nitrogen cycle (Tomura et al. 1994). CYP56 synthesizes *N*, *N*′-bisformyl dityrosine, a component of the outer spore wall layer, by joining the two molecules of *N*-formyl tyrosine (Melo et al. 2008). CYP65, CYP68 and CYP526 are involved in trichothecenes, a group of sesquiterpens (mycotoxins) biosynthesis (reviewed in Kimura et al. 2007). CYP68 is also involved in plant hormone gibberellin biosynthesis (Rojas et al. 2001). CYP532 and CYP535 perform pisatin demethylation, an important

Table 2 Analysis of thermostability of the P450omes of *T. terrestris* and *M. thermophila*

P450	Aliphatic index	Melting temperature (Tm)		Homolog P450 from mesophilic ascomycetes	
		Tm index	Predicted Tm (°C)	Aliphatic index	P450 and species name
T. terrestris					
CYP538A	92.67	−0.1	<55	87.72	CYP538A1 NC
CYP539A	91.17	0.6	55–65	86.63	CYP539A8P AT
CYP643C	93.35	1.4	>65	89.09	CYP643C2 AF
CYP684A	92.63	0.7	55–65	83.71	CYP684A4 AT
CYP5070A	90.83	0.1	55–65	83.37	CYP5070A1 AF
CYP5129A	100.43	1.21	>65	96.08	CYP5129A PN
CYP65AW	92.64	0.78	55–65	88.00	CYP65AW1 FO
CYP53A	95.1	0.8	55–65	85.13	CYP53A1 AN
CYP5249A	91.09	1.17	>65	98.91	CYP5249A HC
CYP5104B	91.33	–	<55	86.22	CYP5104B1 AN
CYP68D	92.31	0.5	55–65	88.31	CYP68D3 AN
CYP5071A	96.83	1.2	>65	88.53	CYP5071A FG
CYP547B	102.34	1.61	>65	100.8	CYP547B3 FO
CYP54A	94.57	1.1	>65	86.05	CYP54A1 ND
M. thermophila					
CYP628C	93.3	0.5	55–65	89.66	CYP628C1 AF
CYP617A	91.12	0.5	55–65	85.34	CYP617A3 FO
CYP547B	101.3	1.07	>65	78.66	CYP547A5 FO
CYP539A	92.33	0.8	55–65	86.63	CYP539A8P AT
CYP5249NS	92.78	1.5	>65	99.84	CYP5249A1P HC
CYP541A	91.88	1.0	55–65	92.89	CYP541A1 NC
CYP606A	97.49	0.7	55–65	86.23	CYP606A3P AT
CYP527A	93.25	0.7	55–65	81.03	CYP527A1 ND
CYP54C	98.56	0.96	55–65	86.88	CYP54C5 FO
CYP660NS	92.21	0.7	55–65	88.24	CYP660A4 AT
CYP61A	96.74	1.3	>65	83.01	CYP61A1 AT
T. thermophilus					
CYP175A1	97.20	1.39	>65		
S. Solfataricus					
CYP119[a]	96.63	0.94	55–65		

Two parameters, *viz*. aliphatic index and protein melting temperature (Tm), were used to measure the thermostablity of P450. P450s that had an aliphatic index of >90 (indicates thermostable P450) were shown in the table along with their Tm. Thermostable P450s' aliphatic index is compared with their homologous P450s from mesophilic ascomycetes (presented as reference species). Ascomycete thermostable P450s' aliphatic index and Tm were also compared with thermostable P450s CYP119 of *S. Solfataricus* and CYP175A1 of *T. thermophilus*. The aliphatic index and Tm are calculated as described in "Materials and methods" section

Abbreviations: PN: *Phaeosphaeria nodorum*; HC: *Histoplasma capsulatum*; AN: *Aspergillus niger*; AF: *Aspergillus flavus*; AT: *Aspergillus terreus*; NC: *Neurospora crassa*; ND: *Neurospora discrete*; FG: *Fusarium graminearum*; FO: *Fusarium oxysporum*; NS: new subfamily

[a] The difference in Tm value predicted by the Tm calculator and wet laboratory experiment (McLean et al. 1998; Yano et al. 2000) is due to the fact that the Tm predictor considers the primary sequence of the protein for measuring the thermostablity. The wet laboratory experiment performed using purified protein involves a three-dimensional structure where the critical amino acids responsible for proteins' thermostablity form a protective fold and increase its thermostablity. This is the critical factor resulting in an increase of Tm in wet laboratory conditions. Despite the difference, the Tm predictor places CYP119 under thermostable P450s

reaction in the detoxification of antifungal toxicants (Maloney and VanEtten 1994). CYP682 is a phenol oxidase involved in the biosynthesis of conidiophore pigment (Mccorkindale et al. 1983). The P450–CPR fusion protein (CYP505) was found to hydroxylate fatty acids (Nakayama et al. 1996), and also to be involved in the synthesis of the mycotoxin fumonisin (Proctor et al. 2003). CYP6001 family P450s are involved in the synthesis of hormone-like, fatty acid-derived oxylipins collectively called "psi" (precocious sexual inducer) signaling factors (Brodhun et al. 2009).

Table 3 Analysis of in vitro stability of the P450omes of *T. terrestris* and *M. thermophila*

P450	Instability index (II)
T. terrestris	
CYP682K	37.99
CYP539A	34.35
CYP643C	31.62
CYP682J	38.57
CYP5129A	36.72
CYP534A	39.17
CYP5249A	37.75
CYP529A	37.99
CYP55A	38.49
CYP559A	35.68
CYP61A	39.73
CYP5071A	39.12
S. solfataricus	
CYP119	39.28
T. thermophilus	
CYP175A1	34.55
M. thermophila	
CYP682N	34.65
CYP534C	33.49
CYP682NS	33.75
CYP547B	35.5
CYP61A	37.23
CYP552C	34.35
CYP548A	37.02
CYP563A	34.39
CYP539A	37.7
CYP541A	36.88
CYP530A	37.96
CYP606A	36.54
CYP559A	39.91
CYP5217A	39.68
CYP52A	38.28
CYP56E	38.51
CYP61A	36.79
CYP533A	38.25
CYP570NS	36.51

The stability of P450s was measured using the Instability index (II) as described in "Materials and methods" section. P450s with an instability index lower than 40 (considered as stable protein) are shown in the table. The instability index of thermostable P450s, CYP119 of *S. solfataricus* and CYP175A1 of *T. thermophilus* is taken as reference

Abbreviation: NS: new subfamily

Transcriptome profiling showed up regulation of several P450s during the fungal growth on different straws as carbon source (Berka et al. 2011). CYP5075NS, CYP586B, CYP526NS, CYP5065A, CYP663A, CYP5249NS and CYP660NS were up-regulated during *M. thermophila* growth on alfalfa straw compared to growth on glucose (Berka et al. 2011). CYP663A also showed up-regulation in response to barley straw. *T. terrestris* P450s, CYP586B and CYP5065A showed up-regulation during growth on alfalfa straw and barley straw compared to growth on glucose. CYP5075A was up-regulated during *T. terrestris* growth on barley straw. Up-regulation of P450s during fungal growth on a straw carbon source suggests the potential role of P450s in wood degradation. However, the specific role(s) of P450s in wood degradation is yet to be identified. Among the P450s (function predicted/known), CYP52A1 from *M. thermophila* is of potential interest, as this P450s showed in vitro stability (Table 3) and can be used for the production of dicarboxylic acids, compounds of potential commercial value (Picataggio et al. 1992).

In this genomic era, bioinformatic programs have become critical in sorting enzymes of potential biotechnological value in an easy and accurate manner. This minimizes laborious and costly laboratory methods of screening etc. Overall, bioinformatic programs are useful to sort potential biotechnologically valuable candidates, but further validation needs wet laboratory experimentation. In this study we performed systematic genome-wide P450 analysis in the two thermophilic biomass-degrading ascomycetes, *T. terrestris* and *M. thermophila,* and identified thermostable P450s using different bioinformatics programs. Based on the aliphatic index, protein melting temperature (Tm) and instability index (II) we identified a large number of thermostable and in vitro stable P450s from two thermophilic ascomycetes. Our results were authenticated by using two well-characterized thermostable P450s, CYP119 and CYP175A1. Six P450s from *T. terrestris* and four from *M. thermophila* are biotechnologically valuable, considering their thermal and in vitro stability. Future work includes experimental characterization of *in silico* predicted thermostable P450s. Furthermore, identified fungal thermostable P450s (this study) can serve as novel tools for understanding the structural and mechanical aspects of protein thermostablity.

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