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Phylogenetic Study of *Penicillium chrysogenum* Based on the Amino Acid Sequence Analysis of Chitin Synthase

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The phylogenetic study of *Penicillium chrysogenum* was performed based on the amino acid sequence comparison of chitin synthase. Phylogenetic trees were constructed with the deduced amino acid sequences of the highly conserved region of chitin synthase gene fragments amplified by PCR. The BlastP similarity search and the bootstrap analysis of the deduced amino acid sequences of chitin synthase from *P. chrysogenum* with those from other fungi showed a close evolutionary relationship of *Penicillium* to ascomycetous fungi, especially to genus *Aspergillus*. The result from bootstrap analysis of the deduced amino acid sequences of the Class II chitin synthase from ascomyceteous fungi supported the usefulness of the Class II chitin synthase for phylogenetic study of filamentous fungi.

Key words: Chitin synthase, *Penicillium chrysogenum*, phylogeny

In the classification of *Penicillium*, the species which form telomorphs were classified in *Eupenicillium* and *Talaromyces*, whereas the strictly anamorphic species were divided into four subgenera-*Aspergilloides, Penicillium, Furcatum*, and *Biverticillium* determined by the type of conidial fruiting structure (17). The subgenus *Penicillium* is by far the most taxonomically difficult subgenus of *Penicillium*, because there are numerous species and the apparent differences are small between species (18). Thus, new approaches were needed to improve the taxonomy and phylogeny of Penicillia.

Molecular techniques for taxonomic and phylogenetic research became developed as means to overcome the difficulties in fungal taxonomy caused by little or no fossil record, lack of a perfect stage, and few available morphological characteristics of fungal taxa (13). One of the recent molecular approaches is the use of the PCR which allows amplication of specific sequences and characterization of fungal species. Identification can thus be achieved through analysis of PCR-amplified products by restriction fragment length polymorphisms, sequencing, or oligonucleotide probing. Most studies aiming at the identification of fungi have been based on the sequence of ribosomal DNA including the internal transcribed space (ITS) region (1, 5, 8, 16).

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Another approach to the identification of fungal species involves the analysis of gene sequences specific for fungi, such as those of chitin synthase encoding genes (*CHS*). Chitin is an important structural polysaccharide of fungal cell walls and septa (3). Comparison of derived amino acid sequences of *Saccharomyces cerevisiae CHS1, CHS2*, and *Candida albicans CHS1* revealed a highly conserved region of about 200 amino acids in length. Short streches of identical amino acid sequences flanking this region are suitable for designing PCR primers. Taking advantage of this, Bowen *et al.* (2) amplified homologous DNA fragments derived from zymogenic chitin synthase-encoding genes of approximately 600 bp obtained primarily from ascomycetous fungi. Multiple sequence alignment of the deduced amino acid sequences from PCR-amplified homologues showed that each homolgues fell into one of three distinct chitin synthase (CHS) classes. Subsequently, this strategy has been used to identify *CHS* homologues from a variety of fungi (9-12. 14, 15, 23). Furthermore, the information obtained from sequence analysis of nucleotides and deduced amino acids is becoming apparently useful in phylogenetic and taxonomic studies of fungi (2, 9).

The analysis of the genes for CHS, therefore, may contribute to the convenience of phylogenetic studies for imperfect fungi like *Penicillium*. We have previously reported on the cloning and the charact-

erization of four CHS gene fragments amplified from the genomic DNA of *Penicillium chrysogenum* which belongs to the subgenus *Penicillium*, using PCR primers designed for highly conserved sequences within fungal CHS encoding genes (14).

In this paper, we report the phylogenetic analysis of the deduced amino acid sequences of CHS from *P. chrysogenum* and discuss the usefulness of the sequence comparison of CHS in taxonomic studies and analyses of evolutionary relationships among the fungi.

Materials and Methods

Source of chitin synthase sequence

The deduced amino acid sequences of CHS from *P. chrysogeum* previously published by the authors (14) (GenBank accession numbers U57321-3 and U 57938) were used in this study. Other deduced amino acid sequences of CHS used for sequence comparison and phylogenetic studies were retrieved from GenBank Nucleotide Sequence Database and PIR Sequence Database through NCBI (Table 1).

Table 1. Taxonomic affinities of fungal species[a], chitin synthase gene designations, and GenBank accession numbers

Division	Class (Subclass)	Order	Family	Species	Gene designatin	GenBank accession No.
Z[b]	Zygomycetes	Entomophthorales	Entomophthoraceae	*Entomophaga aulicae*	*EaCHS2*	L39869
		Mucorales	Mucoraceae	*Rhizomucor circinelloides*	*RcCHS*	X99569
				Rhizopus oligosporus	*RoCHS1*	D10159
				Rhizopus stolonifer	*RsCHS*	X9095
				Phycomyces blakesleeanus	*PbCHS*	L18753
A[c]	Euascomycetes					
	(Discomycetes)	Elaphomycetales		*Elaphomyces muricatus*	*EmCHS*	X78092
	(Plectomycetes)	Eurotiales	Trichomaceae	*Aspergillus (Emericella) nidulans*	*AdCHS1, AdCHS2, AdCHSB*	M82938, M82939, D83216
		Onygenales	Onygenaceae	*Blastomyces (Ajellomyces) dematitidis*	*BdCHS1, BdCHS2*	M92942, M92943
				Histoplasma (Ajellomyces) capsulatum	*HcCHS1, HcCHS2, HcCHS3*	M82947, M82948, M82949
	(Pyrenomycetes)	Phyllachorales	Phyllachoraceae	*Magnaporthe grisea*	*MgCHS1*	X96413
		Sordariales	Sordariaceae	*Nerospora crassa*	*NcCHS, NcCHS3*	M82951, M82950
	Hemiascomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces cerevisiae*	*ScCHS1, ScCHS2*	M14045, M23865
B[d]	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Ustilago maydis*	*UmCHS1, UmCHS2*	M82958, M82959
	Hymenomycetes	Agaricales		*Agaricus bisporus*	*AbCHS*	X98488
		Aphyllophorales	Schizophyllaceae	*Schizophyllum commune*	*SmCHS1*	M82957
M[e]			Mitosporic Trichomaceae	*Aspergillus niger*	*AnCHS, AnCHS2*	M82940, M82941
				Aspergillus fumigatus (Sartorya fumigata)	*SfCHSA, SfCHSB*	S53799, S53800
					SfCHSC, SfCHSG	S53801, G39478
				P. chrysogenum	*PcCHS1, PcCHS2*	U57321, U57322
					PcCHS3, PcCHS4	U57323, U57938
			Mitosporic Onygenaceae	*Cioccidioides immitis*	*CiCHS2*	U60213
		Mitosporic Chaetothyriales		*Exophiala jeanselmei*	*EjCHS2, EjCHS3*	M82945, M82944
				Exophiala (Wangiella) dematitidis	*WdCHS1*	M81905
				Phaeococcus exphiales	*PeCHS2*	M82953
		Mitosporic Saccharomycetales		*Xylophypha batiana*	*XbCHS2*	M82961
				Candida albicans	*CaCHS1, CaCHS2*	M82937, A38192

[a]Taxonomic affinity of species was described according to Taxonomic Scheme of NCBI. [b]Zygomycotina. [c]Ascomycotina. [d]Basidiomycotina. [e]Mitosporic fungi (Deuteromycotina).

Computer analysis

Similarity of deduced amino acid sequences of CHS was determined using the BLASTP program to search the non-redundant PDR+SwissProt+SPupdate+PIR+GenPept+GPupdate database through GenBank. Deduced amino acid sequences of CHS were aligned using the mutiple alignment program CLUSTAL W (22). And then, the dendrogram was constructed with the method of unweighted pair-group arithmetic average (UPGMA) of the software package MEGA version 1.0 (7). A bootstrap analysis of one thousand replications was performed using the heuristic search of MEGA (7). For further study on the deduced amino acid sequences of the Class II CHS, a phylogenetic tree was constructed using PHYLIP package v3.5 (4) and phylogenetic relationships of CHSs were discussed.

Results and Discussion

Similarity with other fungal CHSs

The deduced amino acid sequences from *P. chrysogenum* chitin synthase genes showed higher rate of similarities with those of Ascomycetes and Deuteromycetes being more closely related to Ascomycetes (Table 2). All of the PcCHSs showed more than 90% of similarity to CHSs of Ascomycetes and Deuteromycetes except PcCHS3 which showed 87% similarity with those from Deuteromycetes, while the similarity values to CHSs from Basidiomycetes and Zygomycetes ranged from 70 to 80%. It is also noteworthy that the Deuteromycetes whose CHSs show high levels of similarity to *P. chrysogenum* are known to be closely related to Ascomycetes. *Aspergillus fumigatus* (= *Sartorya fumigata*) which does not produce a telomorph shows clear affinities with the telomorphic genus *Neosartorya* (19). *Aspergillus niger* is a suspected Ascomycetes of the genus *Emericella*. *Phaeococomyces exophilales* is a member of the form family of Fungi Imperfecti which most likely represent loculomycetous Ascomycetes (2). Thus these results may reflect the kin taxonomic relationship between *Penicillium* and ascomycetous fungi.

Multialignment analysis

When the deduced amino acid sequences of the PcCHSs were aligned with those of fungal species whose deduced amino acid sequences could be accessed through GenBank database using CLUSTAL W, they matched well with the three classes of CHS defined by Bowen *et al.* (2). Especially, PcCHS1 revealed characteristic sequence feature of the Class I CHS. PcCHS2 and PcCHS3 revealed those of the Class II CHS. PcCHS4, however, revealed those of the Class III CHS (Fig. 1). Considering the fact that closeness in protein sequences can be interpreted in terms of similarity of function and/or of species (24), the closeness of deduced amino acid sequences between PcCHS2 and PcCHS3 could suggest some reflection on the functional relativeness of the products of two genes or the existence of pseudogene in the Class II CHS. The definitive answers on these issues could be given through the cloning of the cDNA for CHS as well as its genomic equivalent.

Phylogenetic analysis

The UPGMA dendrogram was constructed using the program MEGA to validate classification inferred by the BLASTP and CLUSTAL analyses. One thousand bootstrap replicates were performed and the resulting consensus confirmed our sequence interpretation of the BLASTP and CLUSTAL analyses. As shown in Fig. 2, the chitin syn-

Table 2. Similarity analysis of deduced amino acid sequences of *P. chrysogenum CHS* homologous sequences from other fungi through BlastP[a]

Penicillum chrysogenum gene	Basidiomycetes		Zygomycetes		Ascomycetes		Deuteromycetes	
	Gene	Similarity (%)	Gene	Similarity (%)	Gene	Similarity (%)	Gene	Similarity (%)
PcCHS1	*SmCHS1*	81	*PbCHS*	80	*BdCHS1*	97	*AnCHS1*	97
			RoCHS1	78	*HcCHS1*	97	*StCHSA*	96
PcCHS2	*UnCHS1*	87	*RcCHS*	82	*AdCHS2*	96	*SfCHSB*	96
	SmCHS1	84	*RsCHS*	82	*EmCHS*	93	*AnCHS2*	95
PcCHS3	*UnCHS2*	84	*RoCHS1*	83	*MgCHS1*	95	*PeCHS2*	87
	ScmCHS1	83	*RcCHS*	83	*NcCHS2*	94	*SfCHSb*	87
PcCHS4	*UmCHS1*	81	*EaCHS*	72	*AdCHSB*	98	*SfCHSG*	98
	AbCHS	78	*PbCHS*	71	*HcCHS2*	95	*SfCHSC*	94

[a]For abbreviations and GenBank accession numbers, see Table 1.

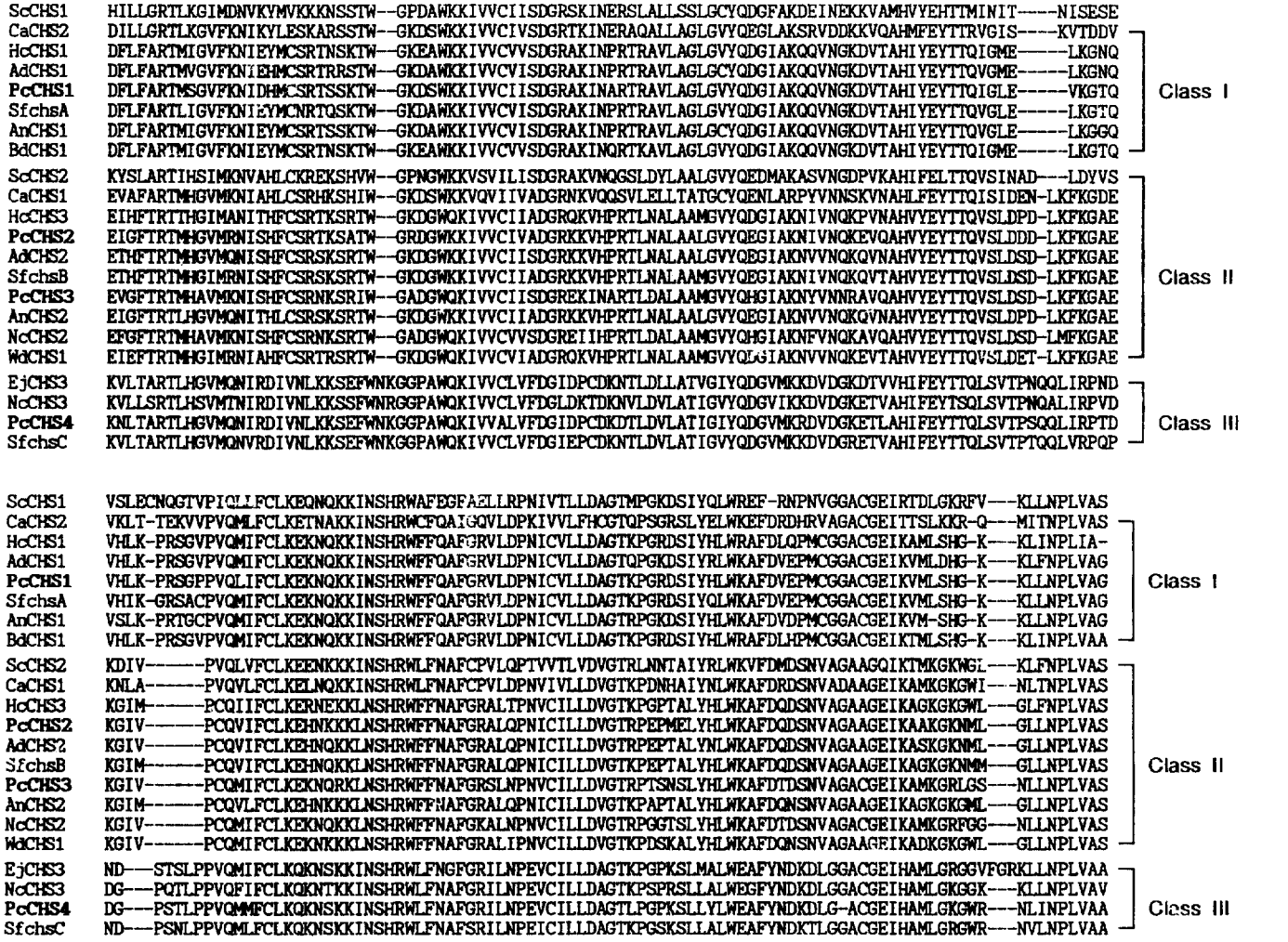

Fig. 1. Multiple amino acid sequence alignment of PcCHSs with the known fungal amino acid sequences which belong to the three classes of CHS. For abbreviations and GenBank accession numbers, see Table 1.

thase classification was similar to that of Bowen *et al.* (2). In addition, the classification of PcCHSs, particularly in the Class I and II, clearly showed that a close relationship between *P. chrysogenum* and *Aspergillus* spp. existed as they were traditionally recognized. In the Class I, *P. chrysogenum* did not cluster with *Histoplasma capsulatus* and *Blastomyces dermatitidis*, which are both ascomycetes of the genus *Ajellomyces*, but clustered with species in the genus *Aspergillus* such as *A. niger, A. nidulans*, and *A. fumigatus*. *Candida albicans* and *S. cerevisiae* were separated from the rest of the fungi investigated as if they were outgroups. In the Class II, PcCHS2 showed the same tendency to the PcCHS1 of the Class I. The additional Class II CHS, PcCHS3, however, did not cluster as the same group with the PcCHS2, but clustered with *Neurospora crassa* CHS2. Assuming that one of the Class II PcCHSs, more probably PcCHS3, is a nonfunctional pseudogene or a functional duplicate of the PcCHS2, the PcCHS3 may be a trait of evolutionary divergency. It may remain even after the separation of the genus *Neurospora* from other groups of genera including *Aspergillus* and *Penicillium*. These speculations are based on several lines of evidences;

1) Among the fungi whose sequences for CHS genes have been identified so far, most of them have only one single copy of gene for the Class II CHS except for the two species belonging to Zygomycetes, *Rhizopus oligosporus* (12) and *Entomophaga aulicae* (23). In the latter case, however, only one of the Class II genes was expressed (23).

2) It was already shown that parsimony analysis of the amino acid sequence of the Class II CHS encoding gene fragments confirmed the current taxonomic grouping (2, 9).

3) The mitochondrial tRNA genes in *P. chrysoge-*

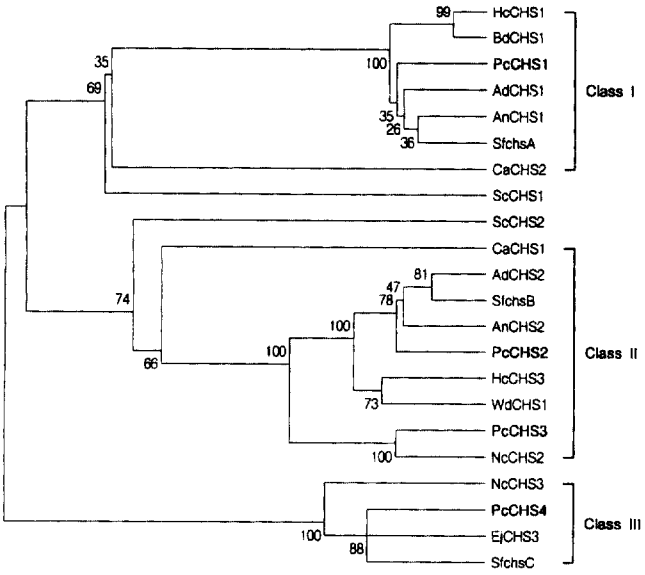

Fig. 2. UPGMA dendrogram showing three distinct chitin synthase classes. The tree was calculated by the program CLUSTAL W from deduced amino acid sequences. The method for estimating the number of amino acid substitutions was *p*-distance. The deduced amino acid sequences of *S. cerevisiae* CHS1 and CHS2 were used as the hypothetical outgroup to construct a parsimonious tree. The numbers are the bootstrap results from 1,000 replications with the heuristic algorithm. For abbreviations and GenBank accession numbers, see Table 1.

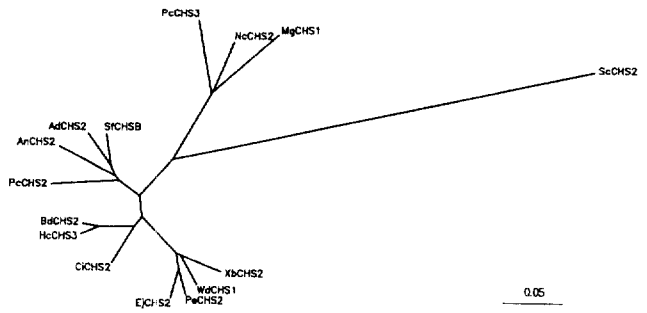

Fig. 3. Tree showing phylogenetic relationships of the Class II chitin synthases. Bootstrap analysis and production of phylogenetic tree of the deduced amino acid sequences of the Class II CHS from ascomycetous fungi were performed using PHYLIP v3. 5. *S. cerevisiae* CHS2 (ScCHS2) was included in the analysis as an outgroup. Branch lengths reflect relative evolutionary distance and are defined by Felsenstein (4). For abbreviations and GenBank accession numbers, see Table 1.

num are strikingly homologous to their counterparts of genomic DNA sequences in *A. nidulans* and *N. crassa* (20).

4) The small subunit of the mitochondrial ribosomal RNA of *P. chrysogenum* is highly homologous (84%) to its counterpart of *A. nidulans* and is 61% or locally more homoglous to that of *Podospora anserina* (21) with *N. crassa* in the family Sordariaceae.

Phylogenetic consideration of the Class II CHS

The unrooted phylogenic tree was constructed with the amino acid sequences of the Class II genes by the program FITCH in PHYLIP to evaluate the usefulness of the Class II CHS comparison in phylogenic studies of filamentous fungi as mentioned previously by others (2, 9). The FITCH analysis of Class II CHS showed quite remarkable clustering of fungi (Fig. 3), traditionally recognized as stated above. The *S. cerevisiae* belongs to the Hemiascomycetes showing considerable evolutionary separation form the Euascomycetes. In Euascomycetes, *N. crassa* and *Magnaporthe griesia* which belong to the Pyrenomycetes are separated from the remaining Euascomycetes. All the mitosporic Chaetothyriales (black yeasts) such as *Exophiala jeanselmei, Phaeococcus exophialae, Wangiella (Exophiala) dermatitidis, and Xylohypha bantiana*, are included in one branch, reflecting their possible plectomycetous affinities. In Plectomycetes, those that belong to the Eurotiales [*A. nidulans, A. niger, A. fumigatus* (=*S. fumigata*), and *P. chrysogenum*] and those that belong to the Onygenales [*Blastomyces* (*Ajellomyces*) *dermatitidis, Histoplasma* (*Ajellomyces*) *capsulatum*, and *Coccidioides immitis*] show clear separation from each other. The tree presented here was, therefore, well congruent with NCBI classification which is one of the major sources for phylogenetic insights derived from molecular evolution studies.

The present results agree with the traditional classifications based on morphological features and also taxonomic and phylogenetic studies based on molecular techniques. We can draw a conclusion that CHSs in fungi reflect the phylogenetic relationships among species and that the analysis of amino acid sequences of chitin synthase genes can be effectively applied for the taxonomic and phylogenetic studies of fungi (2, 9).

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