Comparison of the Genomic Structure of the Heat Shock Protein-88(Hsp88) Genes in the Four Entomopathogenic Fungal Strains, *Paecilomyces tenuipes* Jocheon-1, *P. tenuipes*, *Cordyceps militaris*, and *C. pruinosa*

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Comparison on the genomic structure and phylogenetic relationship of the Hsp88 genes from *P. tenuipes* Jocheon-1, *P. tenuipes*, *C. militaris* and *C. pruinosa* was described. The Hsp88 genes from the three entomopathogenic strains, *P. tenuipes* Jocheon-1(strain), *P. tenuipes*(original species), and *C. militaris* contain the identical genomic structure, namely 5 introns and 6 exons with the length of 13, 62, 32, 1,438, 306, 288 nucleotides encoding 713 amino acid residues, whereas in case of *C. pruinosa*, it contains 4 introns and 5 exons with the length of 13, 62, 32, 1,744, 288 nucleotides encoding 713 amino acid residues. The genomic DNA length of the Hsp88 genes from *P. tenuipes* Jocheon-1 and *P. tenuipes* are both 2,600 nucleotides long in size. The Hsp88 genes from *C. militaris* and *C. pruinosa* are 2,582, 2,576 nucleotides long in size, respectively. Hsp88 genes of the *P. tenuipes* Jocheon-1, *P. tenuipes*, *C. militaris* and *C. pruinosa* also contain the conserved ATP-binding domain. Phylogenetic analysis of the Hsp genes of the four strains tested in this study showed that the fungal Hsp88 is divided into two separate clades, ascomycetes and deuteromycete. Within the ascomycetes fungal clade, the *P. tenuipes* Jocheon-1 and *P. tenuipes* formed a subgroup, on the other hand, *C. militaris* and *C. pruinosa* formed another subgroup. Pair-wise comparison of *P. tenuipes* Jocheon-1 Hsp88 with

those of *P. tenuipes*, *C. militaris* and *C. pruinosa* Hsp88s revealed significant identity in deduced amino acid sequence among these strains. The *P. tenuipes* Jocheon-1 Hsp88 showed 99% identity with the *P. tenuipes*, 97% identity with the *C. militaris*, and 98% identity with the *C. pruinosa*.

Key words: ATP-binding domain, Ascomycetes, Deuteromycete, PCR, Vegetable wasps, Plant worms

Introduction

Vegetable wasps and plant worms are entomopathogenic fungi which attack larva, pupa or adult of host insects, and are known as a mysterious medicine in Asian countries, particularly in China, Korea and Japan from ancient times (Park *et al.*, 2005). In general “vegetable wasps and plant worms” means the fruiting body growing on the larval, pupal or adult integument of the corresponding insect hosts, which is insect-born mushroom showing special appearance produced from the bodies of the host insects attacked by a great number of entomopathogenic fungi (Samson *et al.*, 1988; Shimizu, 1997). In China, vegetable wasps and plant worms have been considered, along with ginseng (*Panax ginseng*) and the young antlers of deer, as one of three oriental medicines that give eternal youth. It has been shown that vegetable wasps and plant worms can produce many kinds of bioactive compounds, and so can be used as functional foods or traditional medicines (Kneifel *et al.*, 1977; Furuya *et al.*, 1983; Montefiori *et al.*, 1989; Yosida *et al.*, 1989; Xu *et al.*, 1992; Fujita *et al.*, 1994; Kuo *et al.*, 2007; Zhu *et al.*, 1998; Nakamura *et al.*, 1999).

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Of the great variety of vegetable wasps and plant worms produced artificially or in native form, *Paecilomyces tenuipes*, *Cordyceps militaris* and *Cordyceps pruinosa* including *Cordyceps sinensis* which is known as the representative “vegetable wasps and plant worms” from the historical view points are the ones that have been investigated extensively, and the potentials for the pharmaceutical uses of which have also been reported. The bioactive compounds from *P. tenuipes* contains cordycepin, sterol, cyclopeptide and the fungus has an inhibitory effect on monoamine oxidase A (MAO-A) and monoamine oxidase B (MAO-B) *in vitro* and *in vivo*, which indicates that it could be considered as a potential antidepressant (Yin *et al.*, 2007). *C. militaris* extract has been reported to exert antitumor activity *in vivo* and *in vitro* (Ohta *et al.*, 2007; Hsu *et al.*, 2008; Park *et al.*, 2009; Zhang *et al.*, 2010; Khan *et al.*, 2010). *C. pruinosa* has been used as traditional folk medicine to treat numerous diseases, and also it was very interesting that it has the potentiality to suppress inflammation (Kim *et al.*, 2010). All cells from simple bacteria to highly differentiated animal cells maintain specialized mechanisms to monitor and respond to their local environmental changes including temperature variation, exposure to toxic compounds, oxidative and reductive stress, ionizing radiation, hypoxia, aging, mechanical injury, and the invasion of pathogens (Davis, 2004). Especially these kinds of environmental changes or stresses mentioned above can induce a cluster of genes (heat shock protein genes; Hsps) encoding heat shock proteins. Heat shock proteins are a specific group of proteins the synthesis of which is induced by heat and a variety of chemical and other environmental stimuli mentioned above. Also, they can be induced in a wide range of organism including bacteria, plants, insects, and mammals (Lemaux *et al.*, 1978; Arrigo *et al.*, 1980; Pelham and Bienz, 1982).

Heat shock proteins were first demonstrated in the fruit fly, *Drosophila melanogaster*, which act as molecular chaperones with essential functions in folding and intracellular translocation of other proteins (Tissieres *et al.*, 1974). The synthesis of heat shock proteins is associated with dramatic effects on the regulation of gene expression, heat shock-related protein synthesis and related differentiation, etc. To understand the full mechanism of the heat shock proteins, the studies on the structure of the genes, biochemistry of the proteins, its function, its transcriptomes, and its proteoms are very important and significant. The major heat shock proteins can be considered in three distinct groups. The first group consists of only one protein hsp83 (also referred to as hsp 82 or hsp 84). It is the most ubiquitous of the heat shock proteins, being synthesized at 25°C in a vari-

ety of cell lines and tissues. It shares homologies with the hsp85 of mammals. The second group of heat shock proteins is the hsp70 group. Hsp70 is often referred to as the major heat shock protein because it is made in the largest amounts and under the most extreme conditions of heat shocks. The third group is the small heat shock proteins, hsp 21~28 (hsp21~28 means the heat shock protein of M.W. 21 kDa~28 kDa) (Peterson and Mitchell, 1985).

For the production of insect mushroom (referred to as silkworm *dongchunghacho*) the entomopathogenic fungi should be inoculated against the silkworm larvae or pupae, and simultaneously heat shock at about 28°C for 24~28 hrs must be performed. During this period, physiologically or pathologically optimal temperatures are different between for the silkworm larvae or pupae and for the fungi itself using as pathogens. At this high temperature of 28°C, the insects are damaged physiologically, but the fungi are not. At this time, we'd like to know inter-relationship of the metabolic phenomena between the insect and the fungi, and it is considered that the key materials would be heat shock proteins for the maintenance of optimally internal condition. For the comparative molecular biological study of the heat shock protein in the insect mushrooms and insects, we had already cloned the cDNA of the heat shock protein 88gene from the entomopathogenic fungi, *P. tenuipes* Jocheon-1 (Liu, 2011), and the current research is focused on the elucidation of genomic structure of the heat shock protein and comparative analysis of its structures based on the phylogenetic viewpoint among the related entomopathogenic fungal species or strains. Especially the present Hsp88 was firstly cloned from *Cordyceps*, and we report. This is the first report of heat shock protein gene in the entomopathogenic fungal strains, *P. tenuipes*, *P. tenuipes* Jocheon-1, *C. militaris* and *C. pruinosa*.

Materials and Methods

Fungus

The four strains of the entomopathogenic fungi tested in the present research are *P. tenuipes* Jocheon-1, *P. tenuipes*, *C. militaris*, and *C. pruinosa*. The fungal strains were artificially cultured in the egg yolk medium as described previously (Lee *et al.*, 2006). *P. tenuipes* is a common fungus which attacks lepidopteran larvae, pupae and adults, and can be found easily from many mountainous areas in Korea (Sung *et al.*, 1997). A method for artificial mass-production of the silkworm vegetable wasps and plant worms (silkworm-dongchunghacho) with the fungal species *P. tenuipes* was established and its many pharmaco-

logical activities were reported in recent years. Also, *P. tenuipes* Jocheon-1 is a strain of the entomopathogenic fungal species *P. tenuipes* which was collected in the mountainous region of Whawang mountain of Changryung-Gun, Korea. The strain showed high infection rate compared to the original species, *P. tenuipes* when the spores were inoculated on the silkworm larvae of host. In addition to that, the newly collected fungal strain showed faster growth for fruiting body formation. The species *C. militaris* was also collected at the Whawangmountain, Changryung-Gun, Korea, the external appearance of the fruiting body shows dense reddish-yellow colour, and the optimal time position for artificial inoculation against the silkworm is just pupal period, on the other hand, for *Paecilomyces* spp. is larval period. These points are of characteristics between the two species. *C. militaris* belongs to ascomycetes, while *P. tenuipes* belongs to deuteromycete. *C. pruinosa* also belongs to ascomycetes, and the fruiting body is especially formed on the lepidopteran silky cocoon (Linacodidae, oriental moth) in nature, in this case the host is also pupae within the cocoon. The species was also collected at the Whawang mountain of Changryung-Gun, Korea, and the red color of fruiting body on the cocoon is of characteristics. More than 350 types (species) of Cordyceps (which are used as general representative name indicating the medicinal mushrooms, *dongchunghacho*) have been reported and discovered until now. Among them, *C. sinensis*, *C. militaris*, *P. tenuipes*, *C. pruinosa* have been used extensively as a crude drug and a folk tonic food in East Asia as described above. The three species (*P. tenuipes*, *C. militaris*, *C. pruinosa*) excluding *C. sinensis* are available as laboratory tools or materials in our lab. Accordingly the three species (total 4 strains) were examined in the present study.

DNA extraction and PCR for the cloning of Hsp88 gene

Genomic DNA was extracted from the fruiting body of *P. tenuipes* Jocheon-1, *P. tenuipes*, *C. militaris* and *C. pruinosa* using a genomic DNA purification kit according to the manufacturer's instructions (Promega, USA). The primers used for amplification of the genomic DNA encoding the Hsp88 were designed; forward primer, named PtHSP-F1, 5'-AGCAACCATGAGTGTCTCGTGTG-3' for the translational start sequence region and reverse primer named PtHSP-R1, 5'-CGTGATATCTATTTGGAATCGGC-3' for the 3' coding region, based on the sequence of *P. tenuipes* Jocheon-1 Hsp88 cDNA sequences cloned in the previous study (Liu, 2011). PCR amplification using these primers and *P. tenuipes* Jocheon-1, *P. tenuipes*, *C. militaris* and *C. pruinosa*

genomic DNAs as the template (50 ng) was carried out using a MyCycler™ Thermal Cycler (Bio-rad, USA) in 20 µl reaction volumes containing 10 pmol each of primer PtHSP-F1 and primer PtHSP-R1, 250 µM dNTP, 10 mM Tris-HCl (pH9.0), 1.5 mM MgCl₂, 30 mM KCl and 1U Taq polymerase. Amplification conditions were: initial denaturation step at 94°C for 7 min, 35 cycle of 94°C for 1 min, 55°C for 1 min, 72°C for 2.5 min and final extension step at 72°C for 7 min. To confirm the successful DNA amplification, electrophoresis was carried out using 0.5× TAE buffer on 1% agarose gel. The PCR product was then purified using PCR purification Kit (QIAGEN, Germany). Each fragment of the gene was cloned into a pGEM-T Easy vector (Promega, USA). For the cloning process, DH5-α competent cells (RBC, USA) were transformed with the ligated DNA, and the resulting plasmid DNA was isolated using a AccuPrepPlasmid Mini Extraction Kit (Bioneer, Korea).

DNA sequencing and data analysis

The sequencing for the cloned fragments of Hsp88 genes from the four entomopathogenic fungi was conducted using the ABI PRISM BigDye Terminator ver. 3.1 Cycle Sequencing Kit with an ABI 377 Genetic Analyzer (PE Applied Biosystems, USA). All products were sequenced from both strands, and the resulting sequences performed by the machine were analyzed among the species or strains of the fungi. In addition, the amino acid sequences of them have also been compared. The sequences were aligned in CLUSTAL W programs (Thompson *et al.*, 1994). Molecular evolutionary genetics analysis (MEGA) software was used for statistical and phylogenetic analysis (Kumar *et al.*, 1994). The phylogenetic tree was constructed by the neighbor joining method (Saitou and Nei, 1987). The GenBank accession numbers of these sequences are as follows: *P. tenuipes*Jocheon-1(GU983957), *P. tenuipes*(HQ822263), *C. militaris*(HQ323694), *C. pruinosa*(HQ323695), *N. haematococca*(EEU34379), *G. graminicola* (EFQ27583), *N. crassa*(AAC23862), *M. oryzae* (EDJ96308), *B. fuckeliana*(EDN30735), *S. sclerotiorum*(EDN96960), *A. dermatitidis*(EEQ88845), *A. benhamiae*(EFQ98775), *T. stipitatus*(EED21621), *P. marneffei* (EEA25736), *Cn var. neoformans*(AAW46676), *L. bicolor* (EDR11689).

Results

Genomic structure of the Hsp88 genes

In order to identify the genomic structure of the Hsp88 genes of *P. tenuipes* Jocheon-1, *P. tenuipes*, *C. militaris* and *C. pruinosa*, a primer set designed based on the

Fig. 1. Continued

Fig. 1. Nucleotide sequence and genomic organization of *P. tenuipes* Jocheon-1 Hsp88 gene. (A) Nucleotide sequence of *P. tenuipes* Jocheon-1 Hsp88 gene. Nucleotide numbers are presented on the left, and the first base of initiation codon of the ORF is defined as +1. The start codon of ATG is boxed and the termination condon is shown by asterisk. The genomic sequence has been provided with the accession number GU983957 from GenBank. (B) Exon/intron structure. Numbers indicate the positions of nucleotides in the genomic sequence of the gene. (C) Lengths of exons, and the summary of the corresponding exon/intron boundaries were given.

sequence information of the *P. tenuipes* Jocheon-1 Hsp88 cDNA already cloned previously as described in introduction (Liu, 2011) was used for cloning of the genomic DNA from these four strains. From comparison of the genomic structure in Hsp88 genes between *P. tenuipes* Jocheon-1 and *P. tenuipes*, it was shown that the two strains belonging to the same species have the same genomic structure; six exons, five introns, and 2,600 nucleotides long in size. Accordingly, the coding sequence between the strains is interrupted by five introns and contains 6 exons with the length of 13, 62, 32, 1,438, 306, 288 nucleotides respectively (Fig. 1 and 2).

Fig. 2. Continued

Fig. 2. Nucleotide sequence and genomic organization of *P. tenuipes* Hsp88 gene. (A) Nucleotide sequence of *P. tenuipes* Hsp88 gene. The genomic sequence has been provided with the accession number HQ822263 from GenBank. (B) Exon/intron structure. (C) Lengths of exons, and the summary of the corresponding exon/intron boundaries were given. For further legends of (A), (B) and (C) see Fig. 1.

In case of *C. militaris*, the genomic sequence is 2,582 nucleotides long in size and the coding sequence is interrupted by five introns and contains 6 exons with the length of 13, 62, 32, 1,438, 306, 288 nucleotides respectively (Fig. 3). The genomic DNA of Hsp88 gene from *C. pruinosa* is 2,576 nucleotides long in size and the coding sequence is interrupted by four introns and contains 5 exons with the length of 13, 62, 32, 1,744, 288 nucleotides, respectively (Fig. 4).

The accession number for the *P. tenuipes*, *C. militaris*, and *C. pruinosa* is HQ822263, HQ323694, and

Fig. 3. Continued

HQ323695, respectively. Comparison of the genomic DNA structure of the *P. tenuipes*, *C. militaris*, *C. pruinosa* with the *P. tenuipes* Jocheon-1 reveals that the Hsp88 coding sequences of the four strains tested are all composed of 713 amino acid residues. Besides, Hsp88 genomic DNAs of *P. tenuipes* Jocheon-1, *P. tenuipes*, and *C. militaris* are composed of 6 exons and 5 introns while Hsp88 genomic DNA of *C. pruinosa* is composed of 5 exons and 4 introns.

Comparison of the amino acid sequences of the Hsp88 genes

Comparison of the sequences of the four entomopatho-

Fig. 3. Nucleotide sequence and genomic organization of *C. militaris* HSP88 gene. (A) Nucleotide sequence of *C. militaris* HSP88 gene. The genomic sequence has been provided with the accession number HQ323694 from GenBank. (B) Exon/intron structure. (C) Lengths of exons, and the summary of the corresponding exon/intron boundaries were given. For further legends of (A), (B) and (C) see Fig. 1.

Fig. 4. Continued

Fig. 4. Nucleotide sequence and genomic organization of *C. pruinosa* Hsp88 gene. (A) Nucleotide sequence of *C. pruinosa* Hsp88 gene. The genomic sequence has been provided with the accession number HQ323695 from GenBank. (B) Exon/intron structure. (C) Lengths of exons, and the summary of the corresponding exon/intron boundaries were given. For further legends of (A), (B) and (C) see Fig. 1.

genic fungal strains, *P. tenuipes* Jocheon-1, *P. tenuipes*, *C. militaris* and *C. pruinosa*, revealed that the Hsp88 gene of all the four strains also contain the conserved ATP-binding domain (Fig. 5). The conserved DXG of PHOSPHATE-1, the CONNECT-1 sequence (E/D)XXXA, the DXG of PHOSPHATE-2, the GS of ADENOSINE and the conserved G within the CONNECT-2 motif (Plesofsky-Vig and Brambl 1998) are all present in all the four amino acid sequences.

Comparison of the amino acid of these four strains revealed that they all contain the same number of amino acid residues (713 amino acid residues) in spite of the

		P1	
<i>P. tenuipes</i> Jocheon-1		MSVVGVDFTGLKTVIAVARNRGVDVINEVSNRSTPSLVGFGPKSRYLGGETAKTQEISNI	60
<i>P. tenuipes</i>		MSVVGVDFTGLKTVIAVARNRGVDVINEVSNRSTPSLVGFGPKSRYLGGETAKTQEISNI	60
<i>C. militaris</i>		MSVVGVDFTGLKTVIAVARNRGVDVINEVSNRSTPSLVGFGPKSRYLGGETAKTQEISNI	60
<i>C. pruinosa</i>		MSVVGVDFTGLKTVIAVARNRGVDVINEVSNRSTPSLVGFGPKSRYLGGETAKTQEISNI	60
<i>P. tenuipes</i> Jocheon-1		KNTVNCCLKRLAGRAFNDPDVQIEQQFITAPLVDDINGQVGAESYLGKQERFTATQVLGMY	120
<i>P. tenuipes</i>		KNTVNCCLKRLAGRAFNDPDVQIEQQFITAPLVDDINGQVGAESYLGKQERFTATQVLGMY	120
<i>C. militaris</i>		KNTVNCCLKRLAGRAFNDPDVQIEQQFITAPLVDDINGQVGAESYLGKQERFTATQVLGMY	120
<i>C. pruinosa</i>		KNTVNCCLKRLAGRAFNDPDVQIEQQFITAPLVDDINGQVGAESYLGKQERFTATQVLGMY	120
		C1	
<i>P. tenuipes</i> Jocheon-1		LSKIKQTAANELKLPVSDLCMSVFFWFTDAQRRALLDASELAGLKLRLINDNTAAALGW	180
<i>P. tenuipes</i>		LSKIKQTAANELKLPVSDLCMSVFFWFTDAQRRALLDASELAGLKLRLINDNTAAALGW	180
<i>C. militaris</i>		LSKIKQTTANERKLPVSDLCMSVFFWFTDAQRRALLDASELAGLKLRLINDNTAAALGW	180
<i>C. pruinosa</i>		LSKIKQTTANERKLPVSDLCMSVFFWFTDAQRRALLDASELAGLKLRLINDNTAAALGW	180
		P2	
<i>P. tenuipes</i> Jocheon-1		GITKLDLPATEEPFKRVFFIDIGHSNYTCISIVEFKKGELAVKATANDRNFGGRDFDKALV	240
<i>P. tenuipes</i>		GITKLDLPATEEPFKRVFFIDIGHSNYTCISIVEFKKGELAVKATANDRNFGGRDFDKALV	240
<i>C. militaris</i>		GITKLDLPATEEPFKRVFFIDIGHSNYTCISIVEFKKGELAVKATANDRNFGGRDFDKALV	240
<i>C. pruinosa</i>		GITKLDLPATEEPFKRVFFIDIGHSNYTCISIVEFKKGELAVKATANDRNFGGRDFDKALV	240
		C2	
<i>P. tenuipes</i> Jocheon-1		DYLAKEFKTKYNADIYTHGRAMARTIAAAEKCKKVL SANQCSAVNIESLMNDIDVSANVT	300
<i>P. tenuipes</i>		DYLAKEFKTKYNADIYTHGRAMARTIAAAEKCKKVL SANQCSAVNIESLMNDIDVSANVT	300
<i>C. militaris</i>		DYLAKEFKTKYNADIYTHGRAMARTIAAAEKCKKVL SANQCSAVNIESLMNDIDVSANVT	300
<i>C. pruinosa</i>		DYLAKEFKTKYNADIYTHGRAMARTIAAAEKCKKVL SANQCSAVNIESLMNDIDVSANVT	300
		A	
<i>P. tenuipes</i> Jocheon-1		RQDFEEMIQLLNRTFEFLEAALAQAFLTKEDIDIVEIVGGGSRVPALKERLQSFFGKPL	360
<i>P. tenuipes</i>		RQDFEEMIQLLNRTFEFLEAALAQAFLTKEDIDIVEIVGGGSRVPALKERLQSFFGKPL	360
<i>C. militaris</i>		RQDFEEMIQLLNRTFEFLEAALAQAFLTKEDIDIVEIVGGGSRVPALKERLQSFFGKPL	360
<i>C. pruinosa</i>		RQDFEEMIQLLNRTFEFLEAALAQAFLTKEDIDIVEIVGGGSRVPALKERLQSFFGKPL	360
		C2	
<i>P. tenuipes</i> Jocheon-1		SYTMNADEAIARGCAFS CAILSPVFNVRDFAVQDIVSYPIEFTWEKAADIPDEDTSLTVF	420
<i>P. tenuipes</i>		SYTMNADEAIARGCAFS CAILSPVFNVRDFAVQDIVSYPIEFTWEKAADIPDEDTSLTVF	420
<i>C. militaris</i>		SYTMNADEAIARGCAFS CAILSPVFNVRDFAVQDIVSYPIEFTWEKAADIPDEDTSLTVF	420
<i>C. pruinosa</i>		SYTMNADEAIARGCAFS CAILSPVFNVRDFAVQDIVSYPIEFTWEKAADIPDEDTSLTVF	420
		C2	
<i>P. tenuipes</i> Jocheon-1		NKGGLLPSTKILTFFYRKQPYDLEARYAKPEDLPKGQNFWMIGRFSVKGVKPTETDDFMVCR	480
<i>P. tenuipes</i>		NKGGLLPSTKILTFFYRKQPYDLEARYAKPEDLPKGQNFWMIGRFSVKGVKPTETDDFMVCR	480
<i>C. militaris</i>		NKGGLLPSTKILTFFYRKQPYDLEARYAKPEDLPKGQNFWMIGRFSVKGVKPTETDDFMVCR	480
<i>C. pruinosa</i>		NKGGLLPSTKILTFFYRKQPYDLEARYAKPEDLPKGQNFWMIGRFSVKGVKPTETDDFMVCR	480
		C2	
<i>P. tenuipes</i> Jocheon-1		LKTRVNIHGILNVETGYHVVEQVEEEVKEDGDFCAMDTDKKDDAPKKTRKVKKQVRAGE	540
<i>P. tenuipes</i>		LKTRVNIHGILNVETGYHVVEQVEEEVKEDGDFCAMDTDKKDDAPKKTRKVKKQVRAGE	540
<i>C. militaris</i>		LKTRVNIHGILNVETGYHVVEQVEEEVKEDGDFCAMDTDKKDDAPKKTRKVKKQVRAGE	540
<i>C. pruinosa</i>		LKTRVNIHGILNVETGYHVVEQVEEEVKEDGDFCAMDTDKKDDAPKKTRKVKKQVRAGE	540
		C2	
<i>P. tenuipes</i> Jocheon-1		LPISSGTSSLDATRIALAEKAAVNEDKLVADTDEKKNLEAYIYDLRSKLEEQYADI	600
<i>P. tenuipes</i>		LPISSGTSSLDATRIALAEKAAVNEDKLVADTDEKKNLEAYIYDLRSKLEEQYADI	600
<i>C. militaris</i>		LPISSGTSSLDATRIALAEKAAVNEDKLVADTDEKKNLEAYIYDLRSKLEEQYADI	600
<i>C. pruinosa</i>		LPISSGTSSLDATRIALAEKAAVNEDKLVADTDEKKNLEAYIYDLRSKLEEQYADI	600
		C2	
<i>P. tenuipes</i> Jocheon-1		ASSEEEKIRAKLEASEDNWLYDEGEDASKGVYIAKIDEIRAMAGPVPVQRHFKEVAEERQV	660
<i>P. tenuipes</i>		ASSEEEKIRAKLEASEDNWLYDEGEDASKGVYIAKIDEIRAMAGPVPVQRHFKEVAEERQV	660
<i>C. militaris</i>		ASSEEEKIRAKLEASEDNWLYDEGEDASKGVYIAKIDEIRAMAGPVPVQRHFKEVAEERQV	660
<i>C. pruinosa</i>		ASSEEEKIRAKLEASEDNWLYDEGEDASKGVYIAKIDEIRAMAGPVPVQRHFKEVAEERQV	660
		A	
<i>P. tenuipes</i> Jocheon-1		ICERYEAEQAARKRAADEEARRAAEAEKAAEGGADAEMKDADGAPAEADAADS	713
<i>P. tenuipes</i>		ICERYEAEQAARKRAADEEARRAAEAEKAAEGGADAEMKDADGAPAEADAADS	713
<i>C. militaris</i>		ICERYEAEQAARKRAADEEARRAAEAEKAAEGGADAEMKDADGAPAEADAADS	713
<i>C. pruinosa</i>		ICERYEAEQAARKRAADEEARRAAEAEKAAEGGADAEMKDADGAPAEADAADS	713

Fig. 5. ATPase domain of Hsp88 from *P. tenuipes* Jocheon-1, *P. tenuipes*, *C. militaris* and *C. pruinosa*. P1, phosphate-1; P2, phosphate-2; C1, connect-1(interdomain hinge); C2, connect-2 (interdomain hinge); A, adenosine.

variation of genomic structure (Table 1). Pair-wise comparison of *P. tenuipes* Jocheon-1 Hsp88 with those of *P. tenuipes*, *C. militaris* and *C. pruinosa* revealed significant identity in deduced amino acid sequences among these species or strains. The *P. tenuipes* Jocheon-1 Hsp88 showed 99% identity with the *P. tenuipes*, 97% identity

with the *C. militaris*, and 98% identity with the *C. pruinosa*(Table 2).

Phylogenetic analysis using the Hsp88 genes

A phylogenetic analysis using the deduced amino acid sequences of known fungal Hsp88 genes revealed that the

Table 1. Comparison of the total number of the each deduced amino acid residues in the Hsp88 genes among the four entomopathogenic fungi tested

Fungi species	A. a. R ¹⁾	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
<i>P. tenuipes</i> Jocheon-1		85	7	56	66	25	40	5	36	58	55	13	25	28	25	39	35	38	52	6	19	713
<i>P. tenuipes</i>		85	7	57	65	25	40	5	36	58	55	13	25	28	25	40	35	37	52	6	19	713
<i>C. militaris</i>		82	8	54	69	26	40	5	39	57	52	13	26	29	24	37	36	38	53	6	19	713
<i>C. pruinosa</i>		82	8	54	68	26	40	5	38	57	53	13	26	28	24	37	36	39	54	6	19	713

¹⁾ Amino acid residues

Table 2. Pairwise identities and similarities of the amino acid sequences of the four entomopathogenic fungi tested and other fungal Hsp88 genes

	Identity percent(%)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. <i>P. tenuipes</i> Jocheon-1		99	97	98	77	76	71	71	69	69	65	67	67	64	46	45
2. <i>P. tenuipes</i>	99		97	97	77	76	71	71	69	69	64	66	66	64	46	45
3. <i>C. militaris</i>	99	98		99	75	75	70	71	68	68	64	66	66	63	45	45
4. <i>C. pruinosa</i>	99	99	99		75	75	71	71	68	68	64	66	66	63	45	45
5. <i>N. haematococca</i>	87	87	87	87		78	71	73	69	69	65	66	67	65	47	46
6. <i>G. graminicola</i>	89	89	89	89	88		75	77	74	74	67	67	69	66	48	47
7. <i>N. crassa</i>	85	85	85	85	84	88		76	71	71	66	68	67	66	49	48
8. <i>M. oryzae</i>	85	85	85	85	84	89	87		71	72	66	67	69	67	48	47
9. <i>B. fuckeliana</i>	85	85	83	84	83	87	86	86		96	70	68	70	69	50	49
10. <i>S. sclerotiorum</i>	84	84	84	83	82	86	86	85	98		70	69	70	69	50	50
11. <i>A. dermatitidis</i>	82	82	81	82	80	82	83	82	83	83		74	73	76	49	48
12. <i>T. stipitatus</i>	83	83	83	83	82	84	84	83	84	84	87		91	72	49	47
13. <i>P. marneffei</i>	83	83	83	82	81	83	83	84	84	84	87	96		74	50	48
14. <i>A. benhamiae</i>	80	80	80	80	79	81	81	82	84	83	86	85	87		49	48
15. <i>Cn Var. neoformans</i>	63	63	64	64	65	66	66	67	66	66	65	67	66	65		64
16. <i>L. bicolor</i>	64	64	63	63	65	65	65	67	67	68	66	66	66	66	79	
Similarity percent(%)																

fungal Hsp88 is divided into two separate clades, ascomycetes and basidiomycetes (Fig. 6). Within the ascomycetes fungal clade the *P. tenuipes*-Jocheon1 and *P. tenuipes* formed a subgroup while *C. militaris* and *C. pruinosa* formed another one.

Discussion

The genomic structure and phylogenetic relationship of Hsp88 genes from *P. tenuipes* Jocheon-1, *P. tenuipes*, *C. militaris* and *C. pruinosa* are described. This is the first report on exon/intron structure of Hsp88 genes in the four corresponding entomopathogenic fungi. The Hsp88 genomic DNAs from *P. tenuipes* Jocheon-1, *P. tenuipes*,

C. militaris, and *C. pruinosa* were cloned by PCR primer set based on the sequences of the *P. tenuipes* Jocheon-1 Hsp88 cDNA sequence cloned previously (Liu, 2011).

In general, eukaryotic heat-shock genes are not interrupted by introns (Lindquist, 1986). However, more recent studies have reported the presence of four introns in Hsp70 gene of the filamentous fungi *N. crassa* (Kapoor *et al.*, 1995) and one intron in aquatic fungus *Blastocladiella emersonii* (Stefani and Gomes, 1995). Introns have been also found in Hsp83 of *Drosophila* (Holmgren *et al.*, 1979) and Hsp70 of *Caenorhabditis elegans* (Snutch *et al.*, 1988). The Hsp88 genomic DNAs from *P. tenuipes* Jocheon-1, *P. tenuipes* and *C. militaris* all contain 5 introns and 6 exons with the length of 13, 62, 32, 1,438, 306, 288 bp, encoding 713 amino acid residues. From the

Fig. 6. Phylogenetic tree of aligned amino acid sequences of the Hsp88 from *P. tenuipes* Jocheon-1, *P. tenuipes*, *C. militaris*, *C. pruinosa* and the other known fungal Hsp88 sequences. The accession numbers of the sequences in the GenBank are as followings: *P. tenuipes* Jocheon-1 (GU983957), *P. tenuipes* (HQ822263), *C. militaris* (HQ323694), *C. pruinosa* (HQ323695), *N. haematococca* (EEU34379), *G. graminicola* (EFQ27583), *N. crassa* (AAC23862), *M. oryzae* (EDJ96308), *B. fuckeliana* (EDN30735), *S. sclerotiorum* (EDN96960), *A. dermatitidis* (EEQ88845), *A. benhamiae* (EFQ98775), *T. stipitatus* (EED21621), *P. marneffe* (EEA25736), *Cn var. neoformans* (AAW46676), *L. bicolor* (EDR11689).

analysis of genomic structure of these Hsp88 genes in 3 strains, almost same size of genomic DNAs (*P. tenuipes* Jocheon-1, 2,600bp; *P. tenuipes*, 2,600bp; *C. militaris*, 2,582bp) were observed even though they are different strains or different species. Also the species showed same number of exons (6 exons) and introns (5 introns), and same number of deduced amino acid residues (713 amino acids) among them. However, in case of *C. pruinosa*, the fourth and fifth exons which exist in Hsp88 genomic DNA of *P. tenuipes* Jocheon-1, *P. tenuipes* and *C. militaris* lacks in *C. pruinosa*. *C. pruinosa* Hsp88 genomic DNA contains 4 introns and 5 exons encoding 713 amino acids. The length of each exon of *C. pruinosa* Hsp88 is 13, 62, 32, 1,744, 288 and the length of exon 4 is identical to the total length of exon 4 plus exon 5 of Hsp88 of *P. tenuipes* Jocheon-1 and *P. tenuipes*. This result is very interesting, but it is also a little bit difficult to considerate or elucidate the reason why.

It was reported that the ATPase domains of Hsp70, actin, and hexokinase have a common three-dimensional structure (Flaherty *et al.*, 1991) and within the structurally similar domains, five sequence motifs named PHOSPHATE-1, CONNECT-1, PHOSPHATE-2, ADENOSINE, and CONNECT-2, have been identified (Bork *et al.*, 1992). The PHOSPHATE-1, PHOSPHATE-2 and ADENOSINE are involved in ATP binding while CONNECT-

1 and CONNECT-2 are involved in the inter domain coupling (Buchberger *et al.*, 1994). Comparison of the sequence of *P. tenuipes*, *C. militaris* and *C. pruinosa* with that of *P. tenuipes* Jocheon-1 reveals that Hsp88 of the *P. tenuipes*, *C. militaris* and *C. pruinosa* also contain the conserved ATP-binding domain (Fig. 5). The conserved DXG of PHOSPHATE-1, the CONNECT-1 sequence (E/D)XXXA, the DXG of PHOSPHATE-2, the GS of ADENOSINE and the conserved G within the CONNECT-2 motif are all present in all the four sequences. Sequence conservation of this sub-domain suggests that the heat shock protein88 retains the Hsp70 mechanism of nucleotide-regulated peptide-binding and release (Plesofsky-Vig and Brambl, 1998).

A phylogenetic tree of known Hsp88 protein sequences was performed. The fungal Hsp88 is divided into two separate clades, ascomycetes and basidiomycetes. Within the ascomycetes fungal clade, the *P. tenuipes* Jocheon-1 and *P. tenuipes* formed a subgroup, on the other hand, *C. militaris* and *C. pruinosa* formed another subgroup. This result shows that the entomopathogenic fungi strains in same genus can be belonged to the same phylogenetic subgroup. Pair-wise comparison of *P. tenuipes* Jocheon-1 Hsp88 with *P. tenuipes*, *C. militaris* and *C. pruinosa* Hsp88 revealed significant identity in deduced amino acid sequence among these strains. The *P. tenuipes* Jocheon-1 Hsp88 showed 99% identity with the *P. tenuipes*, 97% identity with the *C. militaris*, and 98% identity with the *C. pruinosa*. The higher sequence similarity between these fungi indicates a high degree of conservation of the heat shock protein88 through evolution.

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