Taiwania, **44**(4): 491-502, 1999

Variation in Heat Shock Protein Synthesis and Genomic Diversity in the Natural Population of *Aspergillus fumigatus* in Northern Taiwan

Kuei-Yu Chen[1] and Zuei-Ching Chen[2, 3]

(Manuscript received 4 October, 1999; accepted 10 Nomember, 1999)

ABSTRACT: Biodiversity of natural population of *Aspergillus fumigatus* Fres. was investigated from the different localities of northern Taiwan. Three physiotypes of intraspecific diversity of this species were recognized. Accompany to the morphological modification response to thermal stress, each strain of *A. fumigatus* varied significantly to produce heat-shock proteins (HSPs) ranged from 26 - 150 kD. Strain C 8404-9 synthesized six HSPs of 38, 46, 52, 53, 87 and 150 kD at 40℃. Strain C 8404-17 synthesized three HSPs of 26, 28 and 87 kD at 40℃. Strain C 8509-3 synthesized only one HSPs of 87 kD at both 40℃ and 50℃ heat-shock treatments. A HSPs of 87 kD is a common HSPs for *A. fumigatus* and can be a chemotaxonomic marker of this species. RFLP of total DNA digested with *Eco*RI and *Eco*RV could distinguish three physiotypes with the coefficient of similarity ranged from 0.67 to 0.92. It is thus confirmed that there exists intraspecific diversity of *A. fumigatus* in natural population distributed in northern Taiwan.

KEY WORDS: ***Aspergillus fumigatus*, Heat-shock proteins, Genomic diversity, Intraspecific variation, Fungal flora, Taiwan, Fungal ecology.**

INTRODUCTION

Aspergillus fumigatus Fresenius is unique from other species of *Aspergillus* by having not well-formed vesicles, lacking footcells and often with septate conidiophores (Chen and Chen, 1988). It is a very common spoilage mould and usually inhabit in soils as saprophyte. However it infrequently behaves as an opportunistic fungi attacking skins, jaws, bone, lungs or nerve system of warm-blood animals including human beings. Aspergillosis is a representative case in medical mycology caused by this fungus (Hoog and Guarro, 1995; Sutton, *et al.*, 1998; Prescott, *et al.*, 1999). The species is thermotolerant and grows well over a wide range of temperature. It can grow below 20℃ and well at 50℃ (Chen and Chen, 1988; Hoog and Guarro, 1995; Sutton, *et al.*, 1998). The species also produces many active substances or secondary metabolites and mycotoxins such as m-cresol, orcinol, fumigatoxin, spinulosin, 3, 4-dihydroxytoluquinone, tryacidin, fumagillin, ergosterol peroxide, helvolic acid, epoxysuccinic acid, fumitremorgin A & B, tryptoquivaline complex, etc. (Kozakiewicz, 1989). Wide range of its distribution and temperature adaptation provide various ecotypes and physiotypes. In this paper, the intraspecific diversity of *A. fumigatus* in northern Taiwan was investigated with emphasis on the responses to heat-shock and genomic variation.

1. Department of Biology, Chinese Culture University, Taipei 113, Taiwan.
2. Department of Botany, National Taiwan University, Taipei 106, Taiwan.
3. Corresponding author.

MATERIALS AND METHODS

Isolation, Identification and Morphological Observation

Four grams of soil samples were treated in 60℃ hot water for 15 min. About 0.5 gram of this sample was dissolved in 1000 ml of Difico potato-dextrose agar (PDA) or Difco cornmeal agar (CMA) containing chloramphenicol (100mg/l) and rose bengal (0.05 g/l) and incubated at 50℃. Four isolates were obtained from the surface soils of farm land and forest soil of northern Taipei (Fig. 1). The pure cultures of each isolate were grown at 20, 25, 30, 35, 40, and 50 ℃, respectively for the growth test. Identification and morphological observation were carried out by light microscopy and scanning electron microscopy (Chen and Chen, 1995, 1996a, b).

Heat-Shock Protein Synthesis

The test fungi involved three isolates of *A. fumigatus* including one typical isolate of *A. fumigatus* (strain C 8404-9), two morphological variants (strains C 8404-17 and C 8509-3) and *Talaromyces emersonii* Stolk (strain C 8410-1). For heat-shock treatment, all isolates were first cultured in a solid culture medium of yeast starch agar (YpSs – 4g yeast extract, 15g soluble starch, 1g K_2HPO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 20g agar, 1000ml distilled water) at 30℃ for three to seven days and then transferred into a 250 ml Erlenmeyer flask containing 150 ml YpSs broth for three or seven days. Aliquots of 5 ml mycelial suspension were added to an Erlenmeyer flask containing 50 ml of YpSs broth. The cultures were then placed in 30℃, 40℃ and 50℃ water baths for 15 minutes, then 5 μl of ^{35}S-methionine were added to each sample, subsequently returned to their treatment temperature for three hours. After termination of heat shock treatment, all cultures were returned to 30℃ for two hours, and samples were filtered two or three times with phosphate buffer solution (PBS) (0.2 g KCl, 0.8g NaCl, 0.2g KH_2PO_4, 1000ml deionized water) for extraction of heat-shock proteins. If a culture was not immediately used, it was immediately frozen in liquid nitrogen and stored at –70℃.

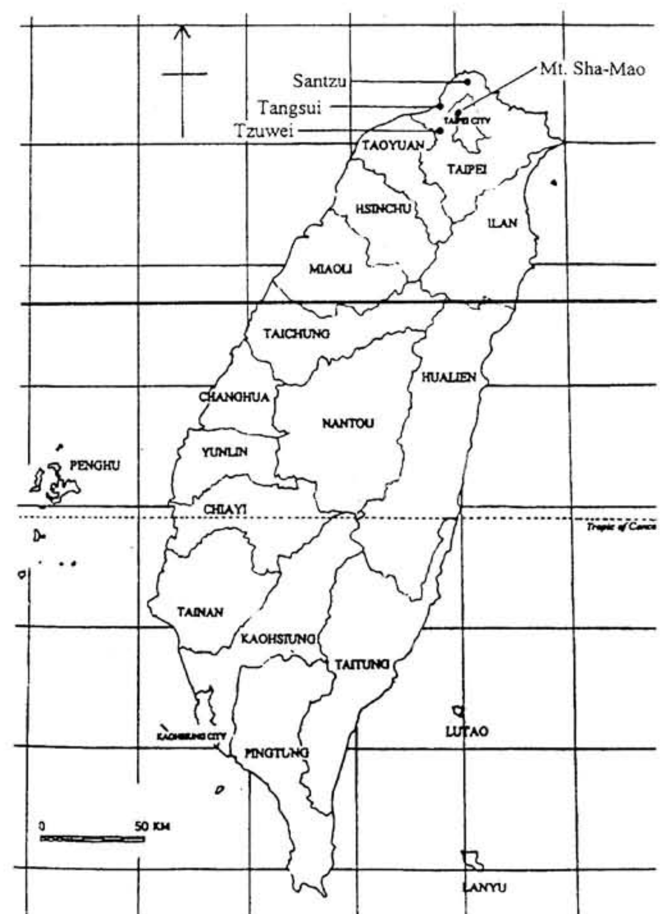


Fig. 1. Sampling area (•) in northern Taipei.

For protein extraction, samples were ground in 1 ml of grinding buffer (50mM Tris/HCl, pH8.5; 2% sodium dodecyl sulfate (SDS); 2% β-mercaptoethanol; 1mM phenylmethy-sulfonylfluoride (PMSF) melted in 95% alcohol) in grinding tube, centrifuged at 12000 rpm

at 28℃ for ten minutes, then supernatant was collected. Four times the volume of cold acetone (-20℃) were added and the samples frozen overnight at -20℃ or for two hours at -70℃. The precipitate (proteins) was stored in acetone, centrifuged at 12000 rpm at 28℃ for 5 minutes, and finally dissolved in 50-100 μl of sample buffer (62.5 mM Tris/HCl, pH 6.8; 3% SDS; 10% glyceral; 5% β-mercaptoethanol). For determination of radioactivity of protein, 5 μl of protein sample was transferred onto a 3 MM filter paper. After drying under infrared light, 10% TCA (trichloroacetic acid) solution was added, boiled in a water bath for three minutes, and the solution discarded. A 5% TCA solution was added again for three minutes, poured out and the sample washed twice in 95% ethanol. Finally, the sample was dried and placed into a counting vial with 6 ml of cocktail (5g 2, 5-diphenyloxazole (PPO) and 0.3g 1, 4-bis-2-15-phenyloxazolybenzene (POPOP)). Radioactivity in CPM was measured in an automatic liquid scintillation counter (Beckman LS 1801). For electrophoresis analysis of proteins, a 12.5% solution of SDS-PAGE acrylamide gel was prepared and the prepared protein sample added. A standard marker was added to the left well, the protein samples were placed in the wells in ascending temperature order and the system run. After electrophoresis, the samples were allowed to solidify. For drying, compression and exposure, the gel was washed one or two times with distilled water, submerged in the solidifying solution (10% acetic acid, 30% methanol), and gently shaken overnight (14-15 hours).The solidifying solution was discarded, EN3HANCE solution (55% acetic acid, 0.4% 2, 5-diphenyloxazol, 15% ethanol, 30% xylene) was added, and the sample gently shaken for one hour. The EN3HANCE solution was discarded and sample softened with 0.8% glycerol for one hour. A gel dryer (Hoefer Scientific Instrument Se 540 Slab Gel Dryer) was used to dry the gel. The dried gel was overlaided with an X-ray film in an exposure chamber at –70℃ for three to fourteen days to allow the radioactive band to appear clearly on the X-ray film. The exposed film was developed with D-19 solution, allowed to dry, and printed. Similarity index between two fungal species is obtained from the following formula (Ludwig and Reynolds, 1988):

Similarity index (%)=(2c/a+b) x 100

a: the number of proteins occurred in species A

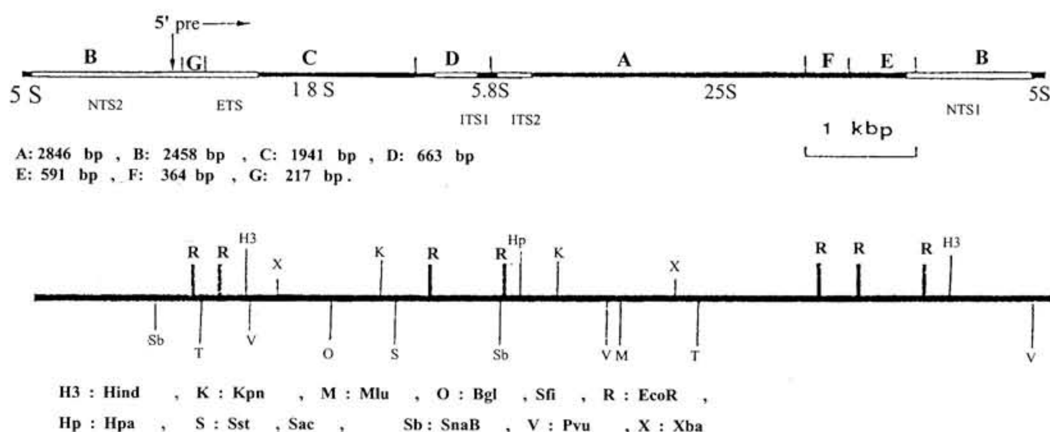
b: the number of proteins occurred in species B

c: the number of common proteins occurred in both species A and B

Restriction fragment length polymorphism (RFLP) of rDNA

All four isolates of *A. fumigatus* were cultured in 150 ml YpSs broth at 40℃ for three to five days by shaking. Genomic DNA was prepared by a " miniprep method " (Lee *et al.*, 1988). Restriction pattern assays were conducted by following procedures. From 0.5-1 μg of DNA was digested with ten units of different restriction enzymes (*Eco*RI, *Eco*RV, and *Hin*d Ⅲ) and subjected to electrophoresis on 0.8 % agarose gels. The gels were blotted onto Hybond Cextra membranes as directed by the manufacturer. The blots were then hybridized to a radioactively labeled DNA probes. Two probes were prepared from eight (A-G) fragments of *Eco*RI digested yeast (*Saccharomyces cerevisiae*) ribosomal DNA repeating unit (Bell, *et al.*, 1977). Fragment A (as Probe A in this experiment) is 2.8 Kb in size, containing one 25 S gene (Fig. 2) and Fragment C (as Probe C) is 1.94 Kb, containing an 18 S gene and ETS region (Fig. 2). Southern hybidizations was carried out after the successful preparation of ^{32}P-labeled probes which were prepared from pBR322 in *Escherichia coli* JM

109. The labeling procedures were following the direction from "Membrane transfer and detection methods" (Amersham, Buckinghamshire, UK). The radioactively labeled blots were then placed on X-ray film(Kodak X-OMAT M20) for autoradiography with intensification screens (Du Pont lighting plus) for one to seven days at –70℃. The numeric analyses of results were followed the method of Moody and Tyler (1990).



From Bell , *et al.* 1977. Ribosomal RNA genes of Saccharomyces cerevisiae I. J. Biol. chem. 252 (22): 8118 -8125.

Fig. 2. Yeast rDNA repeating unit-length 9080 bp.

RESULTS

Intraspecific morphological variations in *A. fumigatus*

Among four strains of *A. fumigatus* isolated form northern Taiwan, strains C 8404-5 and C 8404-9 were identical to the typical *A. fumigatus* according to Raper and Fennell (1965) with dark green , powdery colony morphology in Difico Czapek's agar (CZA), while strains C 8404-17 and C 8509-3 representing pink and brown color variants in culture, respectively (Fig. 3). Two variants showed spherical conidial head below 35℃, instead of typical cylindrical conidial head of this species. At high temperature, over 40-50℃, dome-shaped vesicle changed to the clavate shape without or with sparse or separate phialides (Fig. 4). Conidial morphology were also affected by high temperature, echinulation in conidial surface disappeared and became smooth at 50℃ in strains C 8404-5 and C 8404-9 (Fig. 5), but in strain C 8404-17, echinulation started after nine days at 30-35℃. Strain C 8509-3 produced abundant echinulate conidia at 30-35℃, but turned to smooth at 40℃ (Fig. 6). The morphological and growth variation of four strains of *A. fumigatus* appeared more significant and prominent at 50℃ than 40℃.

Variation in heat-shock proteins (HSPs) synthesis

Responses to heat shock treatment of three isolates of thermotolerant *A. fumigatus* and thermophilic *Talaromyces emersonii* are shown in Figure 7 and Table 1. Except strain C 8509-3 of *A. fumigatus*, no HSP was synthesized at 50℃ treatment. Among three strains of *A. fumigatus*, strain C 8404-9 produced six HSPs (150, 83, 53, 52, 46 and 38 kDs), strain C 8404-17 produced 87, 28, and 26 kDs, strain C 8509-3 synthesized only one HSP of 87 kD

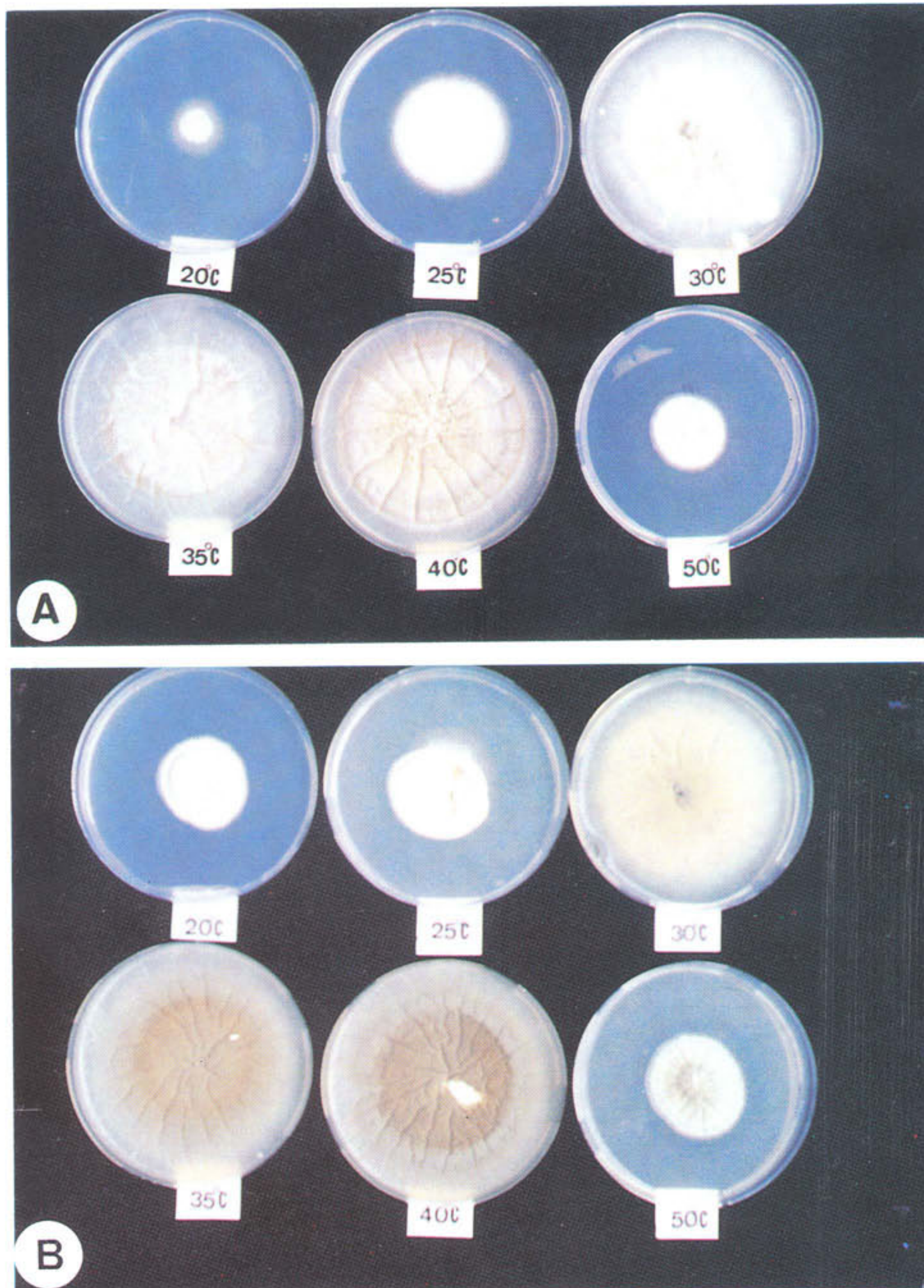


Fig. 3. Cultural morphology of strain C 8404-17 (A) and strain C 8509-3 (B) of *Aspergillus fumigatus*.

in both 40℃ and 50℃ treatment. *T. emersonii* which has *Aspergillus* anamorphic state synthesized six HSPs: 87, 80, 53, 46, 38, and 30 kDs at 40℃ treatment. A heat-shock protein with 87 kD molecular weight seems to be a common HSP of the genus *Aspergillus* and can be a marker of this group. Degree of similarity of HSPs between isolates of *A. fumigatus* and *T. emersonii* as shown in Table 2 indicates that intraspecific variation of HSP synthesis of *A. fumigatus* is larger than interspecific variation between strain C 8404-9 of *A. fumigatus* and *T. emersonii*.

Table 1: Heat Shock Proteins of *Aspergillus fumigatus and Talaromyces emersonii.*

Species	Molecular weight (kD)							
	A2	A3	B2	B3	C2	C3	D2	D3
	150	-	-	-	-	-	-	-
	87	-	87	87	87	-	87	-
	53	-	-	-	-	-	80	-
	52	-	-	-	-	-	53	-
	46	-	-	-	-	-	46	-
	38	-	-	-	-	-	38	-
	-	-	-	-	-	-	30	-
	-	-	-	-	28	-	-	-
	-	-	-	-	26	-	-	-
	-	-	-	-	-	-	-	-

A: *Aspergillus fumigatus* (strain C 8404-9); B: *A. fumigatus* (strain C 8509-3); C: *A. fumigatus* (strain C 8404-17); D: *Talaromyces emersonii* (C 8410-1). 2: Temperature at 40℃; 3: at 50℃.

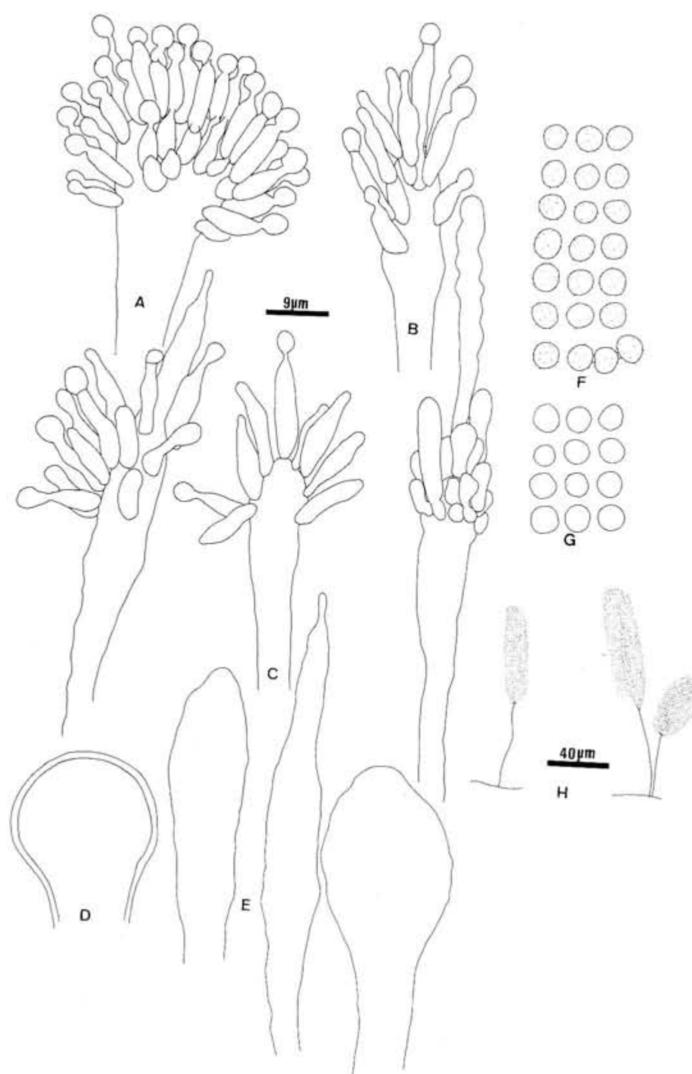


Fig. 4. Effect of temperature on the conidial morphology in strain C 8404-9 of *Aspergillus fumigatus*. A: Conidiophore at 40℃; B-C: Conidiophores at 50℃; D: Vesicle at 40℃; E: Vesicles at 50℃; F: Conidia at 40℃; G: Conidia at 50℃; H: Habit sketches.

Table 2: Degree of similarity of HSPs between species of *Aspergillus fumigatus and Talaromyces emersonii* (%).

Fungal species	A	B	C	D
A	-	25	28.6	66.7
B		-	40	25
C			-	22.2
D				-

A: *Aspergillus fumigatus* (strain C 8404-9); B: *A. fumigatus* (strain C 8509-3); C: *A. fumigatus* (strain C 8404-17); D: *Talaromyces emersonii* (C 8410-1).

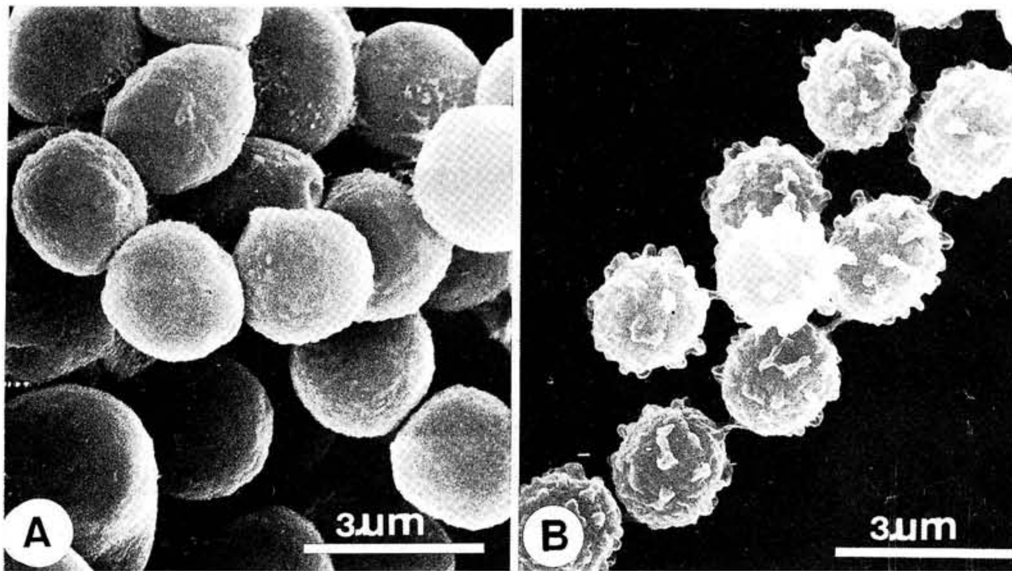


Fig. 5. Effect of temperature on the conidial morphology in strain C 8404-9 of *Aspergillus fumigatus*. A: at 50℃; B: at 40℃.

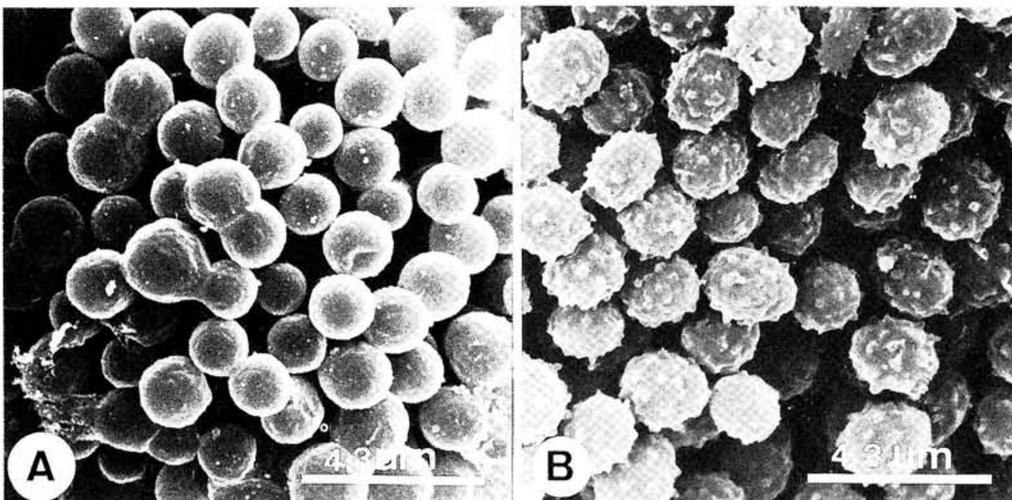


Fig. 6. Effect of temperature on the conidial morphology in strain C 8509-3 of *Aspergillus fumigatus*. A: at 40℃; B: at 25℃.

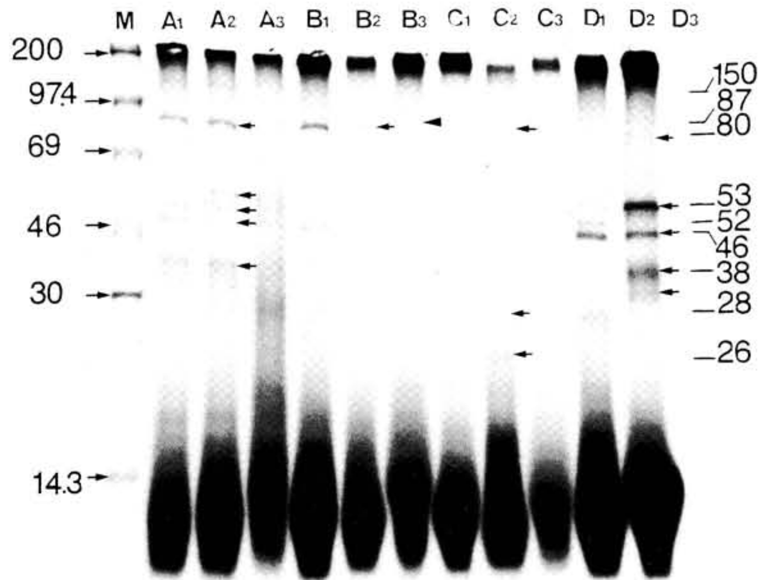


Fig. 7. Synthesis of Heat Shock Proteins by *Aspergillus fumigatus* and *Talaromyces emersonii*. A: *Aspergillus fumigatus* (strain C 8404-9); B: *A. fumigatus* (strain C 8509-3); C: *A. fumigatus* (strain C 8404-17); D: *Talaromyces emersonii* (C 8410-1); M: molecular size marker. 1: at 30℃; 2: at 40℃; 3: at 50℃.

Variation of RFLPs in *A. fumigatus*

The genetic variation of four isolates of *A. fumigatus* detected by RFLP are shown in Figures 8 and 9 as well as in Table 3. The result of this experiment indicates that four isolates are the same species of *A. fumigatus* but strain C 8404-17 shows a slight genetic variation with other strains (Table 4).

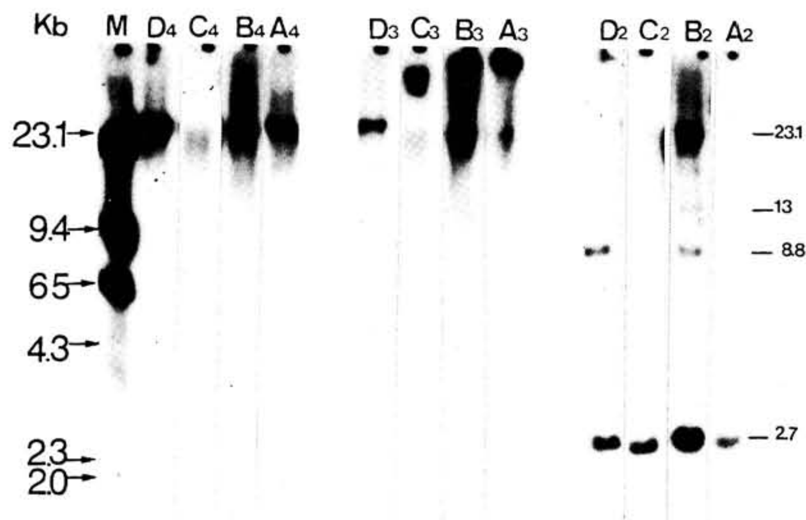


Fig. 8. Result of RFLP of rDNA of *Aspergillus fumigatus* isolates hybridized with probe A and digested with three restriction enzymes *Eco*RI (2), *Eco*RV (3), and *Hind*III (4). M: Lambda DNA / *Hind*III size marker. A: *Aspergillus fumigatus* (strain C 8509-3); B: *A. fumigatus* (strain C 8404-17); C: *A. fumigatus* (strain C 8404-9); D: *A. fumigatus* (strain C 8404-5).

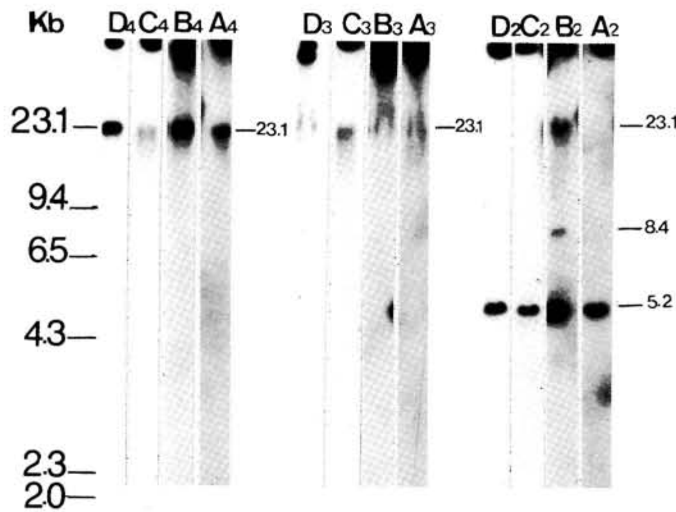


Fig. 9. Result of RFLP of rDNA of *Aspergillus fumigatus* isolates hybridized with probe C and digested with three restriction enzymes *Eco*RI (2), *Eco*RV (3), and *Hin*dIII (4). M: Lambda DNA/ *Hin*dIII size marker. A: *Aspergillus fumigatus* (strain C 8509-3); B: *A. fumigatus* (strain C 8404-17); C: *A. fumigatus* (strain C 8404-9); D: *A. fumigatus* (strain C 8404-5).

Table 3: Result of RFLP of rDNA of four *Aspergillus fumigatus* isolates digested by three restriction enzymes *Eco*RI, *Eco*RV, and *Hin*dIII and hybridized with probe A and C markers ("1" with DNA fragment; "0" without DNA fragment).

		Isolates			
	Molecular size (kb)	A	B	C	D
Probe A					
*Eco*RI	Restriction fragments				
	23.1	0	1	0	0
	13.0	0	1	0	0
	8.8	0	1	0	1
	2.7	1	1	1	1
*Eco*RV	Restriction fragments				
	39.0	0	1	1	0
	23.1	1	1	1	1
*Hin*dIII	Restriction fragments				
	23.1	1	1	1	1
Probe C					
*Eco*RI	Restriction fragments				
	23.1	0	1	0	0
	8.4	0	1	0	0
	5.2	1	1	1	1
*Eco*RV	Restriction fragments				
	23.1	1	1	1	1
*Hin*dIII	Restriction fragments				
	23.1	1	1	1	1

A: *Aspergillus fumigatus* (strain C 8509-3); B: *A. fumigatus* (strain C 8404-17); C: *A. fumigatus* (strain C 8404-9); D: *A. fumigatus* (strain C 8404-5).

Table 4: The coefficient of similarity of DNA fragments of four *Aspergillus fumigatus* isolates digested by three restriction enzymes *Eco*RI, *Eco*RV, and *Hind*III with probe A and C marker.

Fungal species	A	B	C	D
A	-	0.67	0.92	0.92
B		-	0.74	0.74
C			-	0.86
D				-

A: *Aspergillus fumigatus* (strain C 8509-3); B: *A. fumigatus* (strain C 8404-17); C: *A. fumigatus* (strain C 8404-9); D: *A. fumigatus* (strain C 8404-5).

DISCUSSION AND CONCLUSION

A natural population of *A. fumigatus* in northern Taiwan exists undetectable genetic variation except one isolate from Santzu Hsiang (strain C 8404-17). Despite their genetic stability within the species, an intraspecific variation in phenotypic characters were detected. Morphological variation in strains C 8404-17 and C 8509-3 were significant. These two strains differed also in the response to high temperature. The degree of similarity of HSPs synthesis in these two strains indicates that they were closer than the other strains of *A. fumigatus* and *T. emersonii.* It is concluded that within the natural population of *A. fumigatus* in northern Taiwan biodiversity within the species was detected genetically as well as phenotically.

ACKNOWLEDGMENTS

Grateful acknowledgment is made to the National Science Council of the Republic of China for financial support under the following projects: NSC77-0211-B-002-01, NSC77-0606-B-002-167, NSC80-0511-C002-08 and NSC81-0211-B-002-07.

LITERATURE CITED

Bell, G. I., L. J. DeGennaro, D. H. Gelfand, R. J. Bishop, P. Valenzuela and W. J. Rutter. 1977. Ribosomal RNA genes of *Saccharomyces cerevisiae*. J. Biol. Chem. **252**: 8118-8125.

Chen, G.-Y. and Z.-C. Chen. 1988. Thermophilic and thermotolerant fungi in Taiwan (I). Trans. Mycol. Soc. R.O.C. **3**: 1-72.

Chen, K.-Y. and Z.-C. Chen. 1995. Three new records of thermophilic fungi from Taiwan. Taiwania **40**: 127-137.

Chen, K.-Y. and Z.-C. Chen, 1996a. A new species of *Thermoascus* with a *Paecilomyces* anamorph and other thermophilic *Thermoascus* species from Taiwan. Mycotaxon **60**: 225-240.

Chen, K.-Y. and Z.-C. Chen. 1996b. *Thielavia pingtungia* sp. nov., a thermophilic ascomycete from Taiwan. Mycotaxon **60**: 241-247.

Hoog, G. S. and J. Guarro. 1995. Atlas of Clinical Fungi. pp. 140. Centraalbureau voor Schimmelcultures, Baarn and Delft, The Netherlands.

Kozakiewicz, Z. 1989. *Aspergillus* species on stored products. Mycological Papers **161**: 1-188.

Lee, S.-B., M. G. Milgroom and J. W. Taylor. 1988. A rapid, high yield mini-prep method for isolation of genomic DNA from fungi. Fungi Genet. Newsl. **35**: 23-24.

Ludwig, J. A. and J. F. Reynolds. 1988. Statistical Ecology: A Primer on Methods and Computing, pp. 337, John Wiley & Sons. New York.

Moody, S. F. and B. M. Tyler. 1990. Use of nuclear DNA restriction fragment length polymorphisms to analyze the diversity of the *Aspergillus flavus* group: *A. flavus*, *A. parasiticus* and *A. nomius*. Appl. Environ. Microbiol. **56**: 2453-2461.

Prescott, L. M., J. P. Harley and D. A. Klein. 1999. Microbiology. 4th ed. pp. 818. WCB/ McGraw-Hill.

Raper, K. B. and D. I. Fennell. 1965. The genus *Aspergillus*. pp. 242. The Williams & Wilkins Company.

Sutton, D. A., A. W. Fothergill and M. G. Rinaldi. 1998. Guide to Clinically Significant Fungi. pp. 52. Williams & Wilkins.

北臺灣煙麴黴 (*Aspergillus fumigatus*) 種內變異之分析

陳桂玉[1]、陳瑞青[2,3]

（收稿日期：1999 年 10 月 4 日；接受日期：1999 年 11 月 10 日）

摘　　　要

北臺灣不同區域內煙麴黴 (*Aspergillus fumigatus* Fres) 生物歧異度之調查。結果獲得三種生理小種。其形態上的差異反應出對熱逆境有不同之表現。每個品系形成熱休克蛋白質的變異範圍為 26-150 kD, 其中 C 8404-9 品系，於 40℃，會合成 26, 28, 52, 53, 87, 150 kD 等六種熱休克蛋白質；C 8404-17 品系，於 40℃，會合成 26, 28, 87 kD；而 C 8509-3 品系，於 40℃及 50℃，僅合成 87 kD。三個品系，均可合成 87 kD 熱休克蛋白質，故熱休克蛋白質 87 kD 可認為 *A. fumigatus* 化學分類之標記蛋白質。若利用限制酶水解基因片段多形性 (RFLPs) 方法研究。以限制脢 *Eco*RI 和 *Eco*RV 水解 *A. fumigatus* 總基因 (total DNA)，分析結果，其相似度範圍為 0.67-0.92。此可證實分佈在北台灣煙麴黴(*A. fumigatus*)之種內歧異度。

關鍵詞: 煙麴黴，熱休克蛋白質，基因歧異度，種內變異，真菌誌，臺灣，真菌生態。

1. 私立中國文化大學生物系。
2. 國立臺灣大學植物系。
3. 通信連絡員。