



Influence of storage conditions on the infestation of *Tyrophagus putrescentiae* and prevalence of mite hypersensitivity in Taiwan

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Abstract

Sensitization to mites is a considerable factor in the development of allergic diseases. Because of its abundance, *Tyrophagus putrescentiae* (Tp) is the predominant storage mite found in home storage rooms, kitchens, and bakeries. Patients allergic to mites might exhibit a severely hypersensitive reaction upon ingesting Tp-contaminated food. The objective of this study was to investigate the rates of Tp contamination in commercial storage products from various areas, storage conditions, and environments in Taiwan. A specific antibody against Tyr p 3, the allergen on Tp, could be used as an indicator to monitor the contamination condition in storage foods. The microscopic mite examination, allergen detection by ELISA and cultured mite chemotaxis were used to evaluate the prevalence of *T. putrescentiae* contamination. Moreover, the IgE responses of patients allergic to mites were examined. We found that pet food and mushrooms were commonly contaminated with Tp, and this was validated through Tyr p 3 concentration and chemotaxis experiments. Tp contamination rates decreased significantly when samples were sealed and stored at a low temperature (< 4 °C), low relative humidity (RH < 60%), or for longer periods at a low temperature. The results of the clinical study indicated that the mites that elicited major positive IgE responses in allergic subjects were *Dermatophagoides pteronyssinus* and *D. farinae*. Thus, people who are sensitized to *D. pteronyssinus* or *D. farinae* might be at risk of a second anaphylactic reaction due to cross-reactivity upon ingestion of Tp-contaminated food. Accordingly, Tp contamination can be prevented by keeping food packages sealed and stored at a low temperature. This prevents the severe allergic reaction caused by the inadvertent ingestion of contaminated food-borne Tp.

Keywords *Tyrophagus putrescentiae* · Storage products · Temperature · Relative humidity · Climate · Allergic reaction

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Introduction

The most prevalent and well-investigated indoor perpetual allergen sources are domestic mites in tropical, subtropical, and humid areas (Bush 2008; Diette et al. 2008). In human dwellings (coverings and rugs), mites can be found in large quantities in kitchen floor dust, sideboards, and storehouses (Arlian 1991). Therefore, mites can be a significant pestilence with economic consequences and cause respiratory allergies in occupationally exposed individuals (Hosen 1976; van Hage-Hamsten et al. 1994; Vieluf et al. 1993).

Approximately 150 storage mite species are known (Smiley 1984), and more than approximately 20 mite species are considered important from a sanitary perspective. Among them, *Tyrophagus putrescentiae* is one of the considerable species in clinical significance, because of its frequent presence in feeds, foods, grains, and mushrooms (Erban et al. 2016a) and the abundance of *T. putrescentiae* in house dust around the tropical and subtropical regions (Yu et al. 2014, 2015), leading to occupational exposure and allergenic diseases.

Tyrophagus putrescentiae is transparent greyish-white and its legs and mandibles are reddish-brown. The lengths of males and females range between 0.33–0.43 and 0.4–0.7 mm, respectively. The mites live between 15 and 18 days in an ideal environment of 23–25 °C and 50–80% relative humidity (RH) (Sanchez-Ramos et al. 2007). Several allergenic components of *T. putrescentiae* have been detected by sera from allergic patients (Arlian et al. 1997; Liao et al. 2010b). Until present, eight allergens of *T. putrescentiae* were identified and approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. The major allergen, Tyr p 2, is a 16 kDa protein that is detected in sera from 80% of sensitized patients (Johansson et al. 1994) and efficiently induces high IgE reactivity in vitro and in vivo (Eriksson et al. 1998, 2001; Johansson et al. 1999). In addition, the high percentage (50%) of IgE-binding component to the allergen Tyr p 3 indicates that this allergen plays an important role in the pathogenesis of IgE-mediated allergic diseases in *T. putrescentiae* (Liao et al. 2009).

Tyrophagus putrescentiae is the major mite found in bakery crops and staple food crops stored in home storage rooms, kitchens, and warehouses eliciting allergic reactions in farmers or bakery workers via inhalation or skin contact (Arlian et al. 1984; Matsumoto et al. 1996; Vidal and Rial 1998). Moreover, patients who ingest *T. putrescentiae*-contaminated food, such as wheat flour products and pancakes, might cause anaphylactic shock (Blanco et al. 1997; Sánchez-Borges et al. 2013).

A previous study indicated that mites can be significantly repressed at an indoor RH of <51% in a temperate climate (Arlian et al. 2001). There are similar studies in other parts of the world, but no similar studies are conducted in Taiwan. Taiwan is an island located on the Tropic of Cancer. The climate in Taiwan includes four distinct seasons, with tropical and temperate climate, a northeast monsoon in the winter, and a special rainy season in the spring. With an RH of >65%, Taiwan's humidity is suitable for mite survival. Therefore, we conducted a comprehensive survey to establish the relationship between *T. putrescentiae* infestation and climate in Taiwan. To prevent severe allergic reaction caused by eating *T. putrescentiae*-contaminated food, we also investigated the risk of contamination of commercial storage products at various storage times and environmental conditions in various regions of Taiwan.

The group 3 allergens of storage mites (*T. putrescentiae* and *Blomia tropicalis*) and dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*) were investigated in a previous

study, showing highly conserved amino acid sequences of enzyme catalytic triad and substrate binding site (Liao et al. 2009). This result suggests that the group 3 allergen in *T. putrescentiae* (Tyr p 3) may act as a major allergen and has limited cross-reactivity with *D. pteronyssinus*, although slightly higher than cross-reactivity with *Blomia*. Despite the various levels of cross-reactivity present in the same group of allergens, we still try to select a suitable antibody against Tyr p 3 with the lowest cross-reaction and the highest specificity for *T. putrescentiae*. So, an antibody against Tyr p 3 was used in the present study to detect *T. putrescentiae* contamination in the survey samples performed by ELISA. Microscopic mite examination, allergen detection by ELISA and cultured-mite chemotaxis were used to evaluate the prevalence of *T. putrescentiae* contamination in this study. Furthermore, we examined the IgE response induced by dust mites (*D. pteronyssinus* and *D. farinae*) and storage mites (*T. putrescentiae*) to compare the severity level of mite-induced sensitization in allergic patients in Taiwan.

Materials and methods

Sample collection

A total of 130 random samples of stored products, including farina (corn flour, barley flour, and potato flour), beans (red bean, mung bean, and soybean), rice (Peng-Lai rice, sushi rice), cookies (soda crackers, sesame crackers), mushrooms, and pet food (rabbit fodder, mice fodder, dog fodder) from three cities including Taipei, Taichung, and Kaohsiung were collected $3 \times (n=3)$. Then, the sampling environment (residence or grocery store), sampling area, RH, temperature, and sample packaging were recorded and analyzed. Samples stored in the refrigerator were excluded from the climate-related analysis.

Mite detection

A standard quantity with 100 g of each survey sample was gathered for sample examination of mite detection. One live mite of *T. putrescentiae* been counted in a stored product was defined as a positive sample in the mite detection, and this positive sample is classified as an infested sample. The samples were observed using a dissecting microscope. The samples contaminated with *T. putrescentiae* were confirmed by using a microneedle to pick mites on a slide with Hoyer's medium [15 g Gum Arabic (cat. no. 51198) to 25 ml H₂O at 60 °C, 100 g chloral hydrate (cat. no. 15307) and 10 g glycerol (cat. no. G5516); Sigma-Aldrich Merck, St. Louis, MO, USA] before placing them in the oven at 45 °C for 3 days. The mite bodies were changed to a transparent formula, and then the mite species were characterized and identified. Other species of mites were recorded in the mite detection but the results are not presented in this study. Due to the foundation sponsorship, this research is focused on the infestation and hypersensitivity prevalence of *T. putrescentiae*.

Species identification

Microscopic examination was used to start the evaluation of *T. putrescentiae* contamination. *Tyrophagus putrescentiae* and other dust mites were identified by morphological characteristics according to Colloff's textbook (Colloff 2009) and other studies (Fain 1990;

Fayaz et al. 2016). Furthermore, molecular identification of *T. putrescentiae* was performed by the genomic analysis of DNA using a PCR-based method. A previous study indicated that the ribosomal second internal transcribed spacer (ITS2) region can be applied as bar-codes to identify various species of astigmatid mites (Yang et al. 2011). In brief, genomic DNA was extracted from mites. Then PCR was carried out in a reaction mixture with a volume of 50 µl containing 1.5 U Pyrobest DNA Polymerase (code no. R005A; TakaRa Bio, Japan), 10×Pyrobest buffer, 10 mM dNTP mixture, 0.5 µM each of the forward and reverse oligonucleotide primers, and 10 ng template DNA. The PCR product, amplicon of the full-length ITS2 region, was amplified by forward primer 5'-CGA CTT TCG AAC GCA TAT TGC-3' and reverse primer 5'-GCT TAA ATT CAG GGG GTA ATC TCG-3'. The molecular identification of *T. putrescentiae* species was performed by sequence analysis of PCR products and confirmed by Basic Local Alignment Search Tool (BLAST) on the website of the National Center for Biotechnology Information (NCBI).

Tyr p 3 antibody preparation and mite allergen detection

The isopropyl β-D-1-thiogalactopyranoside (IPTG) induced 6X-His tagged recombinant Tyr p 3 (rTyr p 3) was purified by nickel-nitrilotriacetic acid agarose metal affinity column chromatography under the native condition and performed by the QIA expression Kit (Qia-gen, Hilden, Germany; cat. no. 32149) according to the manufacturer's instruction for antibody preparation. The anti-Tyr p 3 polyclonal antibody serum was collected from a New Zealand rabbit by subcutaneous injection with rTyr p 3 emulsified with Freund's adjuvant (Liao et al. 2009).

The detection of mite allergen was performed by enzyme-linked immunosorbent assay (ELISA). In brief, 5 g of survey samples including 20 farina samples, 20 bean samples, 20 cookie samples, 10 rice samples, six mushroom samples, and seven pet food samples were stirred with cell lysis buffer (total protein extraction reagent; Thermo Fisher Scientific, Waltham, MA, USA; cat. no. 87792) for 30 min. Two hundred µl of extracted sample was coated on the 96-well microplate at 4 °C for overnight. Microplates washed with phosphate-buffered saline + 1% Tween-20 (PBST) pH 7.2 and then incubated with anti-Tyr p 3 polyclonal antibody. The diluted secondary antibody (1:5000) of goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Millipore, Temecula, CA, USA; cat. no. AP307P) was incubated for 1 h at 37 °C. The colorimetric reaction was presented by 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Thermo Fisher Scientific; cat. no. 34026). Absorbance at 405 nm was determined using a PowerWare-X340 ELISA reader (Bio-TEK, Winooski VT, USA). The reasonable quantitation of Tyr p 3 was ranging from 5 to 1500 ng/ml. The limitation of this allergen detection of Tyr p 3 performed by ELISA was this method must rely on a good quality standard curve. The correlation coefficient values of the standard curve between allergen concentrations and absorbance greater than 0.95 were considered good quality. The allergen concentrations of the test sample must fall within these concentration ranges in order to deem accurate.

Measurements of RH and temperature in microenvironment

The storage conditions of RH and temperature in the microenvironment of sample collection were measured by digital thermometer and hygrometer (Ketotek, Russia; HTC-2). A total of 3× of time points were sampled of each survey sample, the values were recorded and presented as average.

Climate statistics data

The climate statistics data from years 1981 to 2010, including mean relative humidity, mean temperature, and number of days with mean temperature lower than 10 °C or higher than 30 °C, were collected from the Center Weather Bureau of Taiwan.

Mite chemotaxis experiment

In the laboratory, we established a simple device to observe the changes in the number of mites in different storage commodities and exposure times. *Tyrophagus putrescentiae* (0.1 g, about 500 mites) was placed in a box (30×22×15 cm; Fig. 3a), along with a different test sample per box including: farina (n=14), dry green beans (n=3), solid-state dog food (n=5), powder state dog food (n=3), rice (n=3), cookies (n=5), and dried-processed mushrooms (n=7). The same weight (100 g) of each tested sample was separately placed in the experimental box without refreshing and water supply during the experimental period. Mites were cultured in the experimental box for 2, 4, 12, and 24 weeks. Mites crossing over a distance of about 30 cm on the bottom of the experimental box with plastic material was considered as a positive response. The numbers of mites infested in the different test samples were determined.

Patient sera

In this study, a total of 60 serum samples from patients with symptoms of allergy to mites were collected. All the subjects were recruited from the Division of Allergy, Immunology and Rheumatology in the Taichung Veterans General Hospital (TCVGH), Taichung, Taiwan, and suffered from at least one allergic disease such as asthma, rhinitis, atopic dermatitis, or eczema. A total of three serum samples from non-allergic subjects were collected as the negative control, and all of the above blood sample collections were approved by the Institutional Review Board of TCVGH (no. C050110).

ELISA assay for serum samples from allergic patients

IgE antibodies in serum samples collected from allergic patients who lived in an urban area and who were sensitized to mites (*D. pteronyssinus*, *D. farinae* and *T. putrescentiae*) were detected by ELISA. First, 100 µL of coating buffer (0.1 M NaHCO₃, pH 8.4) was added to each well on the 96-well plate to fix *T. putrescentiae* crude extract (5 µg/mL), and then the plate was covered with saran wrap and stored in a 4 °C refrigerator overnight. The next day, the excess antigen was removed and each well washed with 100 µL PBST 3×, before adding 200 µL of 3% skim milk for 1 h at 37 °C for blocking the non-specific antigen binding sites. After 3×PBST washing, 100 µL of diluted (1:5) human sera was added and incubated overnight at 4 °C. Then, 100 µL of diluted secondary antibody (1:1000 dilution) (anti-human IgE HRP) (Thermo Fisher Scientific; cat. no. A18793) was added to each well and maintained at 37 °C for 1 h after 3×PBST washing. Finally, the plate was washed with PBST 3× and 50 µL of tetra-methylethyl benzidine (TMB) (Sigma-Aldrich Merck; cat. no. 860336) was added in the dark for

15 min before stopping the reaction by adding 50 μL of H_2SO_4 to each well. Absorbance at 450 nm was determined by using a PowerWare-X340 ELISA reader.

Statistical analysis

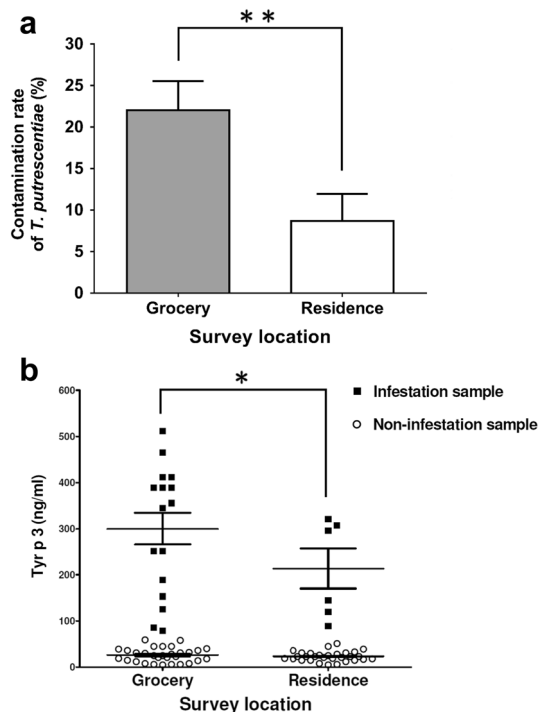
Data regarding different storage categories are represented as the mean $\pm$ SD for each group. The differences between two groups were analyzed with a non-paired t test. The results of contamination of *T. putrescentiae* by microscopic investigation, allergen examination, chemotaxis assay, storage packing conditions (RH and temperature) were analyzed by ANOVA, followed by Tukey's or Dunnett's multiple comparisons tests ($\alpha=0.05$).

Results

Comparison of contamination rate between residences and grocery stores

The *T. putrescentiae* contamination rate might be affected by storage micro-environment. The common types of storage micro-environments can be divided into two broad categories including residence environment and grocery store. Of the 130 survey samples, the average contamination rate in the group from the grocery store was about 22% (14/63), higher than the 9% (6/67) from the residence environment ($P<0.01$) (Fig. 1a). The mite presence in survey samples collected from the residence environment and grocery store was confirmed by the antibody against Tyr p 3. The mean allergen concentrations of Tyr

Fig. 1 Comparison between residence and grocery store product *Tyrophagus putrescentiae* contamination rate based on 130 samples, from 3 collections. **a** Mean ($\pm$ SD) incidence (%) of (live) *T. putrescentiae* infestation based on microscopic examination. **b** Mean ($\pm$ SD) Tyr p 3 concentration (ng/ml). Asterisks indicate significant differences (t test: * $0.01 < P < 0.05$, ** $P < 0.01$)



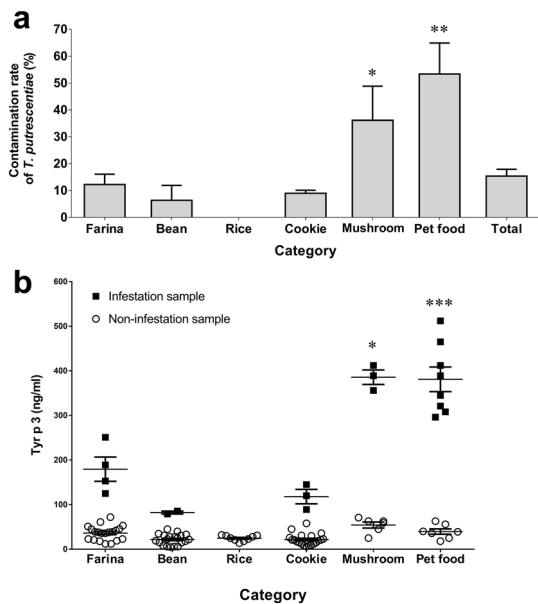
p 3 in samples collected from the grocery stores were higher than from residence environment (301.5 vs. 203.1 ng/ml; $P < 0.05$) (Fig. 1b).

Products contaminated and allergen content in Taiwan

To understand which types of stored items are easily contaminated by *T. putrescentiae* in Taiwan, data on the storage conditions of surveyed samples including farina, beans, rice, cookies, mushrooms, and pet foods were collected and analyzed. In these survey samples, the average positive rate of *T. putrescentiae* contamination in storage products was about 15.4% (20/130). The average positive rates per food category were as follows: farina 12.5% (4/32), beans 6.5% (2/31), rice 0% (0/9), cookies 8.8% (3/34), mushrooms 33.3% (3/9), and pet foods 53.3% (8/15) (Fig. 2a). The results showed that the contamination rate of pet foods was higher than that of mushrooms and farina. In addition, *T. putrescentiae* contamination was rarely observed in stored beans and was absent in rice.

Protein sequence diversities still exist among group 3 mite allergens. Cross-reactivity of group 3 allergens among these species of mite was studied using *T. putrescentiae*-sensitized sera. IgE antibodies against rTyr p 3 were found in 58% (58/100), in 50% (50/100) after absorption with *D. pteronyssinus* and in 38% after absorption with *Blomia* (data shown in Supplementary Fig. 1). In order to confirm the specificity of *T. putrescentiae*, the western blotting of mite crude extracts (*T. putrescentiae* or *B. tropicalis*) was performed by anti-Tyr p 3 antibody recognition. A stronger intensity of the 26-KDa protein reacting with anti-Tyr p 3 antibody was observed in the extracts of *T. putrescentiae* but almost no reaction at *B. tropicalis* (Supplementary Fig. 2). Furthermore, molecular identification of *T. putrescentiae* was performed by the DNA analysis of ITS2 using a PCR-based method. A total of five contaminated samples were randomly selected for molecular identification, the ITS2 DNA sequences were identical with *T. putrescentiae* partial sequence ID: GQ205624.1 on

Fig. 2 *Tyrophagus putrescentiae* contamination rate in various food types (farina, bean, rice, cookie, mushroom, and pet food). **a** Mean ($\pm$ SD) incidence (%) of (live) *T. putrescentiae* infestation based on microscopic examination. **b** Mean ($\pm$ SD) Tyr p 3 concentration (ng/ml). The concentration of Tyr p 3 in non-infested samples was considered as a background value. Asterisks indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test: * $0.01 < P < 0.05$, ** $0.001 < P < 0.01$, *** $P < 0.001$)



the website of NCBI (Supplementary Fig. 3). The species in the survey samples was confirmed by morphological characteristics and molecular identification to be *T. putrescentiae*.

The results of microscopy were validated by specific antibody, anti-Tyr p 3, to detect the allergen content in the survey samples. The allergen concentration in the infested samples was higher than in non-infested samples (Fig. 2b). The mean concentrations of Tyr p 3 in the infested farina, cookie, and bean samples were approximately 185, 120, and 80 ng/ml, respectively. In addition, the Tyr p 3 concentrations were clearly higher in the infested samples of mushroom and pet food with the mean concentrations of Tyr p 3 up to 400 ng/ml. These data were consistent with our microscopic examination.

Validation of contamination

To validate the results of the field study, a chemotaxis experiment was done in the laboratory. An illustration of the experimental setup is shown in Fig. 3a. The results showed that the *T. putrescentiae* contamination rate increased in farina, solid pet food, cookie, powder pet food, and mushrooms after 2, 4, 12, and 24 weeks of incubation (Table 1). Among these items, the contamination rates of *T. putrescentiae* in pet food and mushrooms were higher than those of farina, beans, and rice. After 24 weeks, the *T. putrescentiae* contamination rates of pet foods and mushrooms had reached 100%. Beans and rice were not contaminated with *T. putrescentiae* after 24 weeks exposure (Table 1). These findings reflect

Fig. 3 Chemotaxis experiment of *Tyrophagus putrescentiae*. **a** Illustration of the experimental setup. Mites (0.1 g, about 500 mites) were added to one side of each box and a test sample of various food types was present on the opposite side. **b** Mean ($\pm$ SD) number of mites after 24 weeks, in the various storage categories tested: farina (n = 14 boxes), bean (n = 3), solid pet food (n = 5), powdery pet food (n = 3), rice (n = 3), cookie (n = 5), and mushrooms (n = 7). Asterisks indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test: **** $P < 0.0001$)

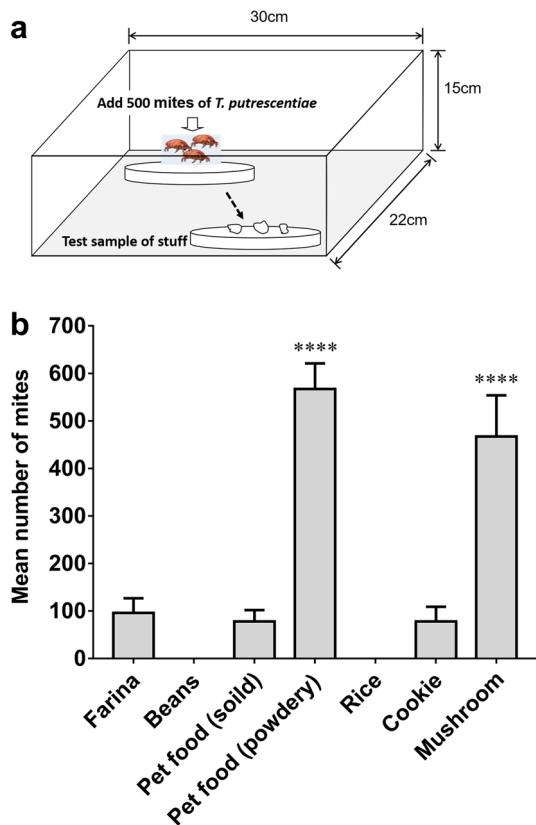


Table 1 *Tyrophagus putrescentiae* contamination rates (%) of various storage products after incubation for 2–24 weeks

Storage product	Incubation time (Weeks)			
	2	4	12	24
Farina	7.1	14.3	35.7	85.1
Beans	0	0	0	0
Pet food (solid)	0	0	33.3	100
Pet food (powdery)	20	100	100	100
Rice	0	0	0	0
Cookies	0	0	20	60
Mushrooms	0	42.9	100	100

the previous results of the field study indicating that stored rice and beans were rarely contaminated with *T. putrescentiae* (Fig. 2a). After 24 weeks of incubation we counted the *T. putrescentiae*. The numbers of mites in the pet food (powdery, but not solid) and mushrooms were significantly higher than in the other products (Fig. 3b).

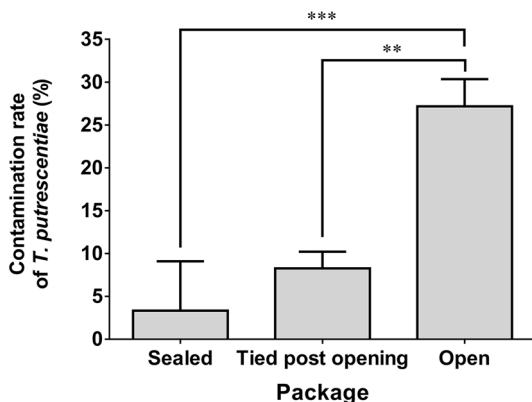
Correlation of storage packing conditions and contamination rate

The *T. putrescentiae* contamination rate might be affected by the packaging conditions. The packing conditions of storage products were classified into three types: sealed, tied after opening, and opened. The average contamination rate of stored samples was about 3.6% (1/28) in the sealed group, 8.5% (4/47) in the tied after opening group, and 27.3% (15/55) in the opened group (Fig. 4). The contamination rate was higher in the opened group than in the sealed groups and in those tied after opening.

Correlation of storage relative humidity and contamination rate

A previous study indicated that maintaining a RH of less than 50% can reduce numbers of house dust mites (Arlian et al. 2001; Qiu et al. 2010). Therefore, the collected RH values in the micro-environments of survey samples were analyzed. The RH values were classified into five levels: 40–50, 50–60, 60–70, 70–80, and > 80%. The average *T. putrescentiae*

Fig. 4 *Tyrophagus putrescentiae* contamination rate in three storage packing conditions: sealed, tied post opening, and opened. Mean (+SD) incidence (%) of (live) *T. putrescentiae* infestation based on microscopic examination. Asterisks indicate significant differences (one-way ANOVA followed by Dunnett's multiple comparison test: **0.001 < P < 0.01, *** P < 0.001)



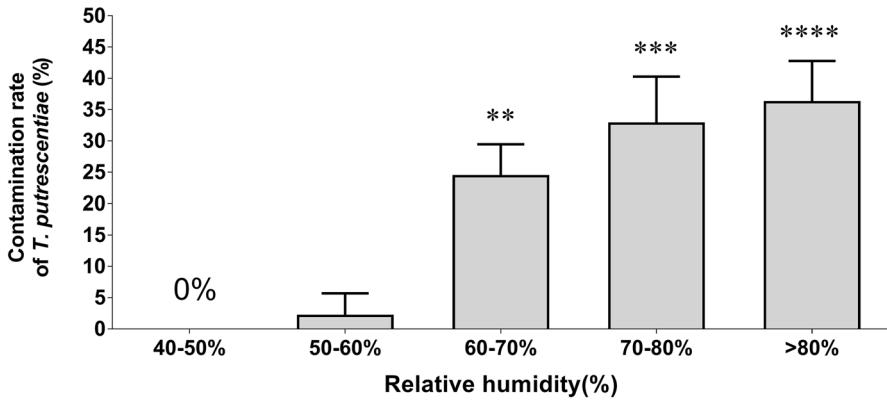


Fig. 5 *Tyrophagus putrescentiae* contamination rate at five levels of relative humidity (RH). Mean (+SD) incidence (%) of (live) *T. putrescentiae* infestation based on microscopic examination. Asterisks indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test: ** $0.001 < P < 0.01$, *** $0.0001 < P < 0.001$, **** $P < 0.0001$)

contamination rates were as follows: 40–50% RH: 0% (0/23); 50–60% RH: 2.4% (1/42); 60–70% RH: 24.2% (8/33); 70–80% RH: 33.3% (5/15), and >80% RH: 35.3% (6/17) (Fig. 5). Hence, when products were stored at >70% RH, the *T. putrescentiae* contamination rate was significantly higher than when products were stored at <60% RH.

Correlation of storage temperature and contamination rate

The storage temperatures of the micro-environment at the time of sample collection were classified into four groups: ca. 4 °C (placed in the refrigerator), 5–15 °C, 16–25 °C, and >25 °C. The average *T. putrescentiae* contamination rates in each group were: ca. 4

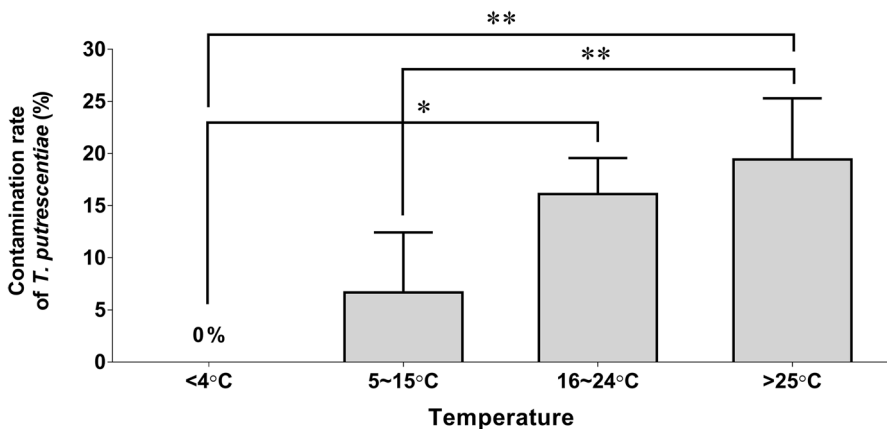


Fig. 6 *Tyrophagus putrescentiae* contamination rate at four levels of storage temperature. Mean (+SD) incidence (%) of (live) *T. putrescentiae* infestation based on microscopic examination. Asterisks indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison test: * $0.01 < P < 0.05$, ** $P < 0.01$)

°C, 0%; 5–15 °C, 8.3% (2/24); 16–25 °C, 16% (8/50); and > 25 °C, 19.6% (10/51) (Fig. 6). The results indicated that when storage products were maintained at > 16 °C, the *T. putrescentiae* contamination rate significantly increased compared with placement at ca. 4 °C.

Analysis of contamination rate in different survey areas

The Tropic of Cancer divides Taiwan into the torrid zone and the temperate zone. Because of the location of the sun directly overhead, the torrid zone typically has a hot and dry climate. The randomly sampled stored samples were analyzed based on whether they were gathered from the temperate zone (Taipei and Taichung) or the torrid zone (Kaohsiung). The samples kept in the refrigerator were excluded. The average *T. putrescentiae* contamination rate of stored samples was about 24.8% (7/28) south of the Tropic of Cancer (torrid zone) and 13.5% (13/97) north of the Tropic of Cancer (temperate zone) (Fig. 7a). According to the statistical data from the Central Weather Bureau of Taiwan, the average temperature, RH, and number of days with high temperature are similar between the torrid and temperate zone (Fig. 7b,c). The numbers of days with temperatures > 30 °C were also similar in both zones (Fig. 7d). However, the number of days in the temperate zone with a minimum temperature below 10 °C was less than that in the torrid zone (Fig. 7e). Hence, the infestation of *T. putrescentiae* may be affected by the number of days with a minimum temperature below 10 °C in natural climates.

Prevalence of mite sensitization in allergic patients

Although a high *T. putrescentiae* contamination rate was observed in products stored in a grocery store (Fig. 1), an urban population inadvertently sensitized to allergens from various mites might be at a higher risk of developing a severe immune response due to secondary exposure upon accidental ingestion of *T. putrescentiae*-contaminated food. Therefore, serum from 60 allergic patients who lived in an urban area and were sensitized to mites (*D. pteronyssinus*, *D. farinae* and *T. putrescentiae*) were collected. The positive rates of IgE reactions to mites from 60 allergic subjects in Taiwan were as follows: 81.7% (49/60) to *D. pteronyssinus*, 75% (45/60) to *D. farinae* and 35% (21/60) to *T. putrescentiae* (Fig. 8). These results indicated that most of the urban allergic patients were more sensitized to house dust mites than to storage mites.

Discussion

Sensitization to mites is a considerable risk factor for the development of allergic diseases such as asthma, allergic rhinitis, and atopic dermatitis. *Tyrophagus putrescentiae* is a familiar storage mite, and it is found in grain, flour, and stored food. In total 130 survey samples were collected in this study; the result indicated that the average contamination rate of *T. putrescentiae* was higher in the grocery store than in the residence environment (22.2 vs. 9%). The properties of different stored products played an important role in affecting the storage mite contamination rate. Although previous research showed that *T. putrescentiae* prefer living in houses over store environments (Matsumoto and Satoh 2004), the results of allergen detection indicated that storage products were at risk for *T. putrescentiae* contamination in Taiwan. Among the survey storage categories, pet food had the highest rate of *T. putrescentiae* and Tyr p 3 contamination, possibly for the following two reasons. One,

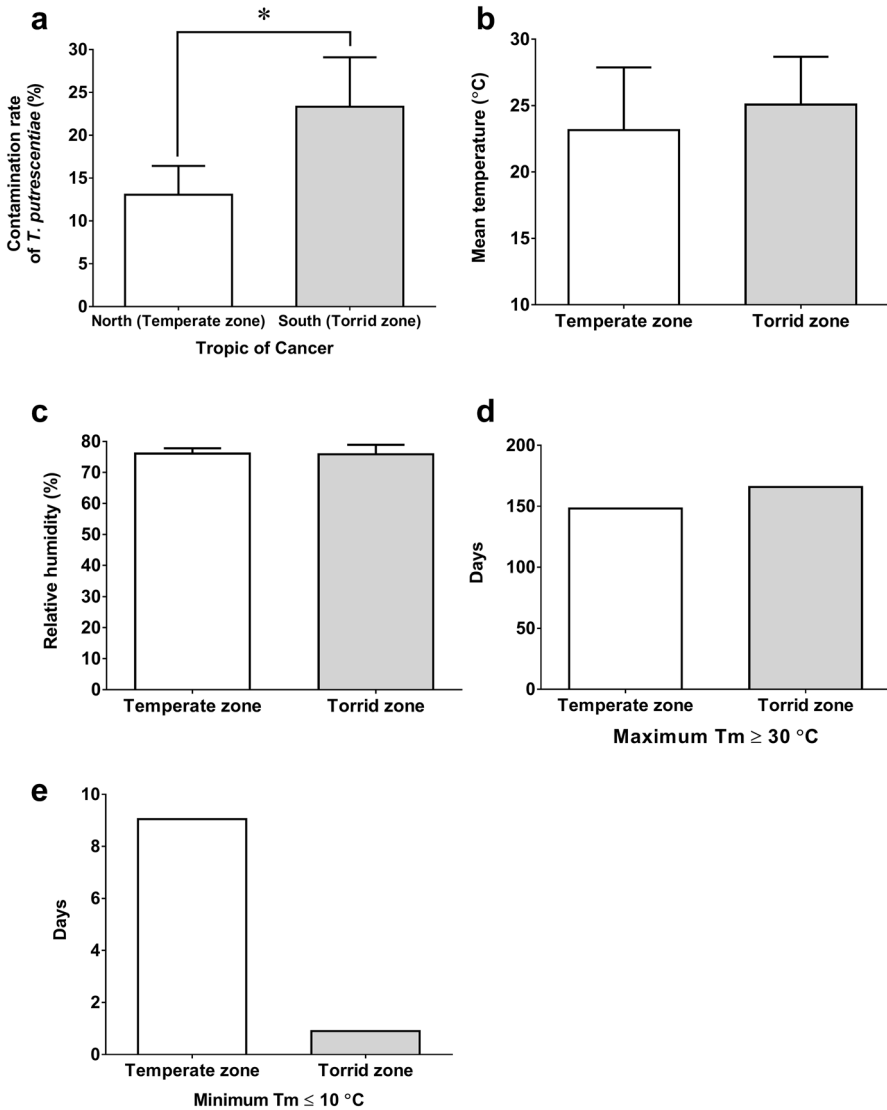
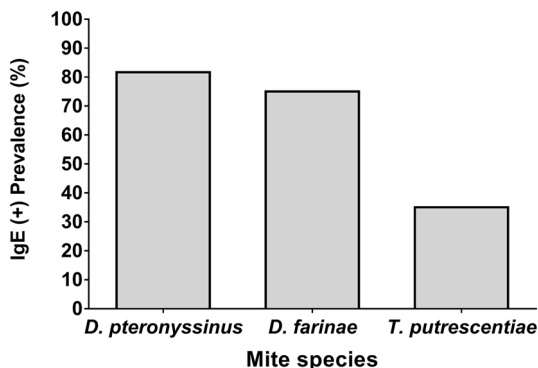


Fig. 7 **a** *Tyrophagus putrescentiae* contamination rate—i.e., mean (+SD) incidence (%) of microscopically examined (live) *T. putrescentiae* infestation in randomly sampled storages from the temperate ($n=100$) and torrid ($n=30$) zones in Taiwan, north and south of the Tropic of Cancer, respectively. Climate statistics comparison of the torrid and temperate zones: **b** mean temperature (°C), **c** relative humidity (%), **d** number of days > 30 °C, and **e** number of days < 10 °C. The asterisk indicates a significant difference (t test: $*P < 0.05$)

the giant packs of pet food (as large as 20 kg) were left open and placed on the floor for a long period of time. The long-time exposure of pet food to the air might raise the risk of *T. putrescentiae* contamination. And two, pet food was always mixed with several kinds of cereals and germ. The mixing process might provide an opportunity for mite contamination. Another study indicated that the environment and storage conditions may influence *T.*

Fig. 8 Prevalence and frequency of IgE binding to allergenic components of mites in allergic patients (n=60), sensitized to *Dermatophagoides pteronyssinus*, *D. farinae* and *Tyrophagus putrescentiae*, as determined by measuring serum IgE antibodies against mite crude extracts



putrescentiae contamination of dog fodder (Brazis et al. 2008). We found that the contamination rates of pet food and mushrooms were higher than of other food types. We speculate the population growth of *T. putrescentiae* is higher on protein- and fat-rich food compared with high-carbohydrate diet (Erban et al. 2015).

The relationship between packing conditions and contamination rate of *T. putrescentiae* was investigated. We found that in the completely opened sample of stored products it was easier to identify mite infestation as compared to the sealed group. Similar findings were reported that the infestation of storage mites in dried fruits and dog feed was presumably caused by inadequate sealing of the packages (Hubert et al. 2015). In the traditional markets of Taiwan large packages of storage products were placed in the grocery stores for a long time. The repeated opening and tying might increase the risk of mite contamination. Thus, we recommend that if a storage product is suitable for *T. putrescentiae* growth, such as mushrooms, it should be tightly packed and stored at a low temperature in a dry micro-environment.

Previous studies indicated that the optimum temperature for *T. putrescentiae* development was 30 °C. Growth of *T. putrescentiae* was inhibited at temperatures above 34.8 °C and below 10.4 °C (Sanchez-Ramos and Castanera 2005). Another study indicated that the optimal growth condition for three species of storage mites—*T. putrescentiae*, *Acarus siro*, and *Lepidoglyphus destructor*—was storage at an RH higher than 85% (Hubert et al. 2013). Our data also indicated that the *T. putrescentiae* contamination rate at a humidity higher than 70% was significantly higher than that at a low RH (under 60%). The optimal relative humidity levels above 60% has been reported to promote the growth of mites on the surface of dry-cured ham. These experiments provided us with a reliable method to address which environment is appropriate for the growth of *T. putrescentiae* (Hendrix et al. 2018). Another study suggested that mushrooms are suitable hosts for *T. putrescentiae* with the temperatures ranging from 16 to 34 °C at 80% RH (Qu et al. 2015). Therefore, the temperature and RH in the chemotaxis experiment were maintained at 25–28 °C and above 60%, respectively.

We found that mushrooms seemed to be highly suitable to support the growth of *T. putrescentiae*. The moist pleating mushrooms with many spores might facilitate *T. putrescentiae* growth and propagation. We also speculated that the repeated dividing from the large package of mushrooms for selling to consumers might result in the high *T. putrescentiae* detection rate. *Tyrophagus putrescentiae* was considered originally to feed on fungi and has subsequently become a serious pest of a wide range of stored food and feedstuffs

including mushrooms. *Tyrophagus putrescentiae* is considered a major pest of mushroom culture with such a wide host and habitat range, its occurrence in mushroom compost and also in fresh and dried mushrooms, and as a potential vector of parasitic fungi in mushroom beds (Kheradmand et al. 2007). Although *T. putrescentiae* are able to withstand a vast range of environmental factors, at lower temperatures they show little ability to reproduce and sustain growth in mushrooms (Kheradmand et al. 2007).

The greater detection of *T. putrescentiae* in grounded powdery compared to solid pet food suggests that the grinding of grain materials may provide a better environment for the growth of *T. putrescentiae*. This would also match the observation that bakers who were sensitized to flour were also sensitized to storage mites (Revsbech and Andersen 1987; Revsbech and Dueholm 1990). The integrity of the kernels in stored products should be maintained because the disrupted or crushed granules promote increases in mite populations (Rybanska et al. 2016). Therefore, the low contamination rates of *T. putrescentiae* in beans and rice might be due to the solid and hard properties of these grains, which do not support the growth and reproduction of *T. putrescentiae*.

Temperatures below 4 °C repress *T. putrescentiae* infestation. Similarly, it was reported that low levels of mite infestation were found in samples packed and stored in the refrigerator (Suesirisawad et al. 2015). According to our results, storing flour products in a low-temperature environment such as in a refrigerator is a good method to avoid allergic symptoms caused by contact with mite-infested storage products.

The analysis of *T. putrescentiae* contamination rate in the torrid vs. temperate zone showed a significantly higher contamination rate in the torrid zone. The number of survey samples is only small and the source of stored products diverse, so we have to be cautious when looking for an explanation. A field investigation for mite detection from 63 townships in Taiwan showed a similar trend that most of the stored grain samples had high mite infestation rates in the southern sections as compared to the other regions of Taiwan (Tseng 2012). Previous studies showed that a low proportion of mite infestation in countries with higher latitudes is related to a lower temperature (Aygün et al. 2007). Taiwanese climate statistics, including average temperature and RH, showed no significant differences between the two zones, so these climate factors cannot explain why the torrid zone obtained higher *T. putrescentiae* contamination rates. Interestingly, the analysis of climate data showed that the number of days with a minimum temperature below 10 °C was higher in the temperate zone than in the torrid zone. Combined with our previous results indicating that low temperature can efficiently inhibit the growth of *T. putrescentiae*, more days with low temperature might be key for repressing the growth of mites in higher altitude areas.

In our previous study, more than 80% of the dust samples contained mites, including *D. pteronyssinus*, *D. farinae* and *T. putrescentiae* (Yu et al. 2014). Because of their abundance and high allergenicity, house dust mites (*D. pteronyssinus* and *D. farinae*) and storage mites (*T. putrescentiae*) are common in infestation and associated with high levels of allergy with airway hypersensitivity (White et al. 2001). In patients sensitized to house dust mites, most were exposed to them by contact with mattresses or carpets in the household environment (Liao et al. 2013). In this study we found that the major predominant mites that elicited positive IgE responses in allergic patients were *D. pteronyssinus* (81.7%) and *D. farinae* (75%); these patients were more sensitized to house dust mites than to the storage mite *T. putrescentiae* (35.0%). A previous study indicated that *T. putrescentiae* hypersensitivity might be due to cross-reactivity with *D. pteronyssinus*, especially in young allergic rhinitis patients (Liao et al. 2010a). Therefore, despite fewer allergic patients being sensitized to *T. putrescentiae*, a severe second anaphylactic reaction might be induced via cross-reactivity

with *D. pteronyssinus* or *D. farinae* in patients who accidentally consume *T. putrescentiae*-contaminated food or products.

Tyrophagus putrescentiae is quite widespread in domestic habitats such as carpets or mattresses in urban environments, farms, the food industry, and even in fungal cultures in laboratories (Duek et al. 2001). Like insects they may derive nutritive advantages from interactions with bacteria and/or both fungi (Douglas 2015). Microorganisms associated with mites can synthesize various nutrients, provide essential amino acids and contribute to digestive processes (Douglas 2015). The bacterial communities were analyzed by Sanger sequencing of 16S rRNA, indicating that the diet and habitats influence not only ingested bacteria but also the symbiotic bacteria of *T. putrescentiae* (Erban et al. 2016a). The distinct difference in bacterial communities among populations of *T. putrescentiae* is presumably due to environmental factors such as geographic origin, microhabitat, and diet (Erban et al. 2016a). Mite populations contain divergent bacterial communities consisting of gut microorganisms and intracellular symbionts and these microorganisms may spread via the mites' fecal pellets. The microbiome profiles of (symbiotic bacteria of) *T. putrescentiae* could be altered by a change in diet (Hubert et al. 2018). Similar diet-related effects may also have played a role in our finding of variation in contamination rates of *T. putrescentiae* among the various food types.

The trypsin-like allergen of *T. putrescentiae*, Tyr p 3, has been identified in the mite feces by 2D-E-MS/MS (two-dimensional gel electrophoresis-mass spectrometry) proteomic analysis and it possessed the highest protease activity when reared on dried dog food (Erban et al. 2016b). Similarly, Der f 3 was detected by 2-DE-MS proteomic approach in feces of *D. farinae* (Erban and Hubert 2015). The authors suggested that the protease allergen Der f 3 identified in the feces remains highly stable after passing through the gut and accumulates in the environment during the life of mites (Erban and Hubert 2015). Differences of fecal allergens/digestive enzymes among fecal extracts of *D. pteronyssinus* and *D. farinae* were investigated using 2D-E coupled with MALDI-TOF/TOF identification—Der p 3 was rarely detected, but Der f 3 exhibited high abundance in mite feces (Erban et al. 2017). Monoclonal antibodies against Der f 3 are strongly immune-positive to posterior midgut mucosa, gut contents and fecal pellets of *D. farinae*, suggesting the trypsin-like allergen of Der f 3 may be synthesized in and secreted from the digestive tract and excreted from the mite's body in the fecal pellets (Zhan et al. 2010). Physiological characteristics and pH of the gut of storage mites are similar to those of house dust mites, indicating that *T. putrescentiae* may possess analogous digestive enzymes and fecal properties (Erban and Hubert 2010). Extracts of *T. putrescentiae* feces contained higher levels of trypsin compared to body extracts, and this protease is involved in the digestive process in mites (Ortego et al. 2000). The trypsin-like serine protease allergen Tyr p 3 can be detected in similar amounts in the crude extracts from mite bodies and spent growth medium (rich with feces) by antibody recognition (Liao et al. 2009). Overall, the protease allergen of Tyr p 3 is present not only in mite body but also in the feces which accumulated in the microenvironment. So, the Tyr p 3 detected in the current study's samples could originate from the mite bodies and feces. It is difficult to distinguish where allergens come from; the high Tyr p 3 allergen contents do confirm contamination of the stored products by *T. putrescentiae*.

Allergen detection of Tyr p 3 performed by ELISA relies on a high-quality standard curve. Experimental samples must be in the linear range of this standard curve, it is inaccurate to speculate in case samples are out of range. The standard curve in this study was made by recombinant protein of group 3 mite allergen (rTyr p 3) expressed in *Escherichia coli* which are not folded exactly the same as native Tyr p 3 (nTyr p 3). The use of recombinant protein with such a different folding structure could lead to inaccurate outcomes.

Therefore it is risky to use this method alone as a diagnostic assay, and we suggest that it can only be used as supporting evidence. Mite identification in this study was based on morphological characteristics and microscopic examination and by DNA analysis of ITS-2.

In this study, we provide evidence that *T. putrescentiae* prefer living in moist or powdery foods such as mushrooms and powdered pet food. Human foods such as rice might not be easily contaminated with *T. putrescentiae*. Moreover, we observed that low temperature, low RH, and package sealing of food can effectively repress the growth of *T. putrescentiae*. Interestingly, we found that the number of days with low temperature in cities with different altitudes might be a relevant factor for reducing *T. putrescentiae* contamination. Most importantly, we found that the patients in the torrid zone or temperate zone with house dust mite allergies might be at risk of developing a severe immune response by ingesting *T. putrescentiae*-contaminated food. Therefore, we suggest enforcing the sealing of food packages and storage at a low temperature or dry environment to inhibit the growth of *T. putrescentiae* and therefore prevent the immune response from accidentally consuming *T. putrescentiae*-contaminated food.

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Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

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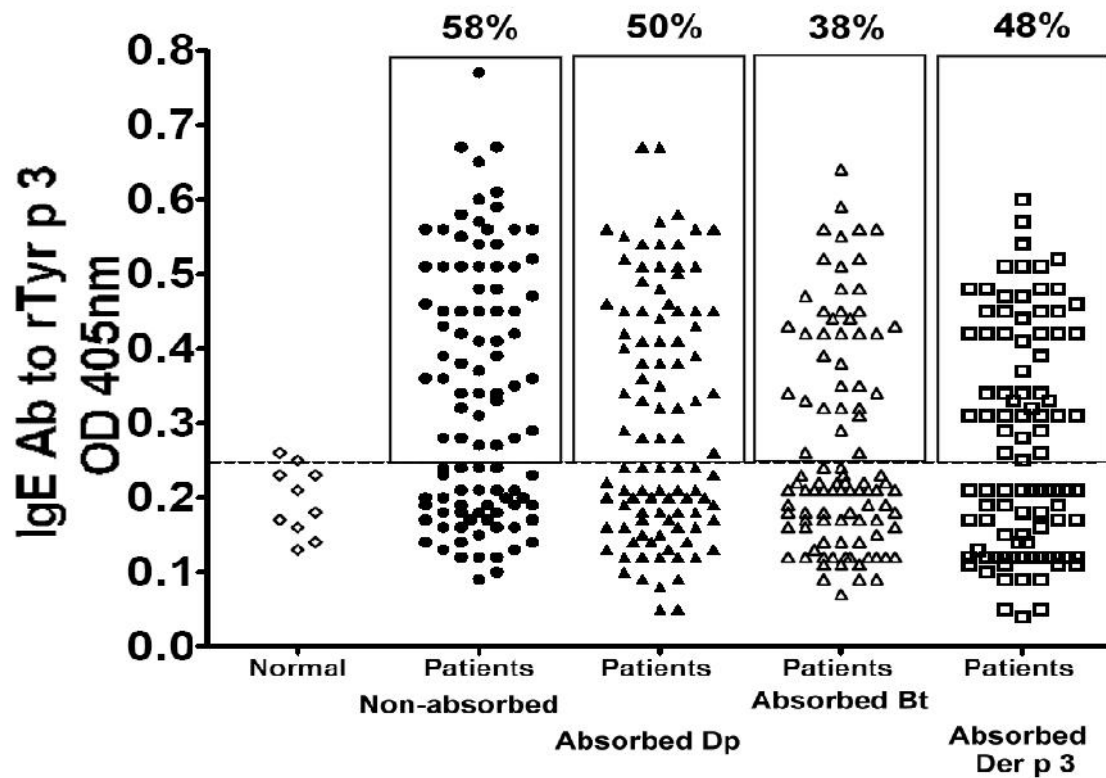
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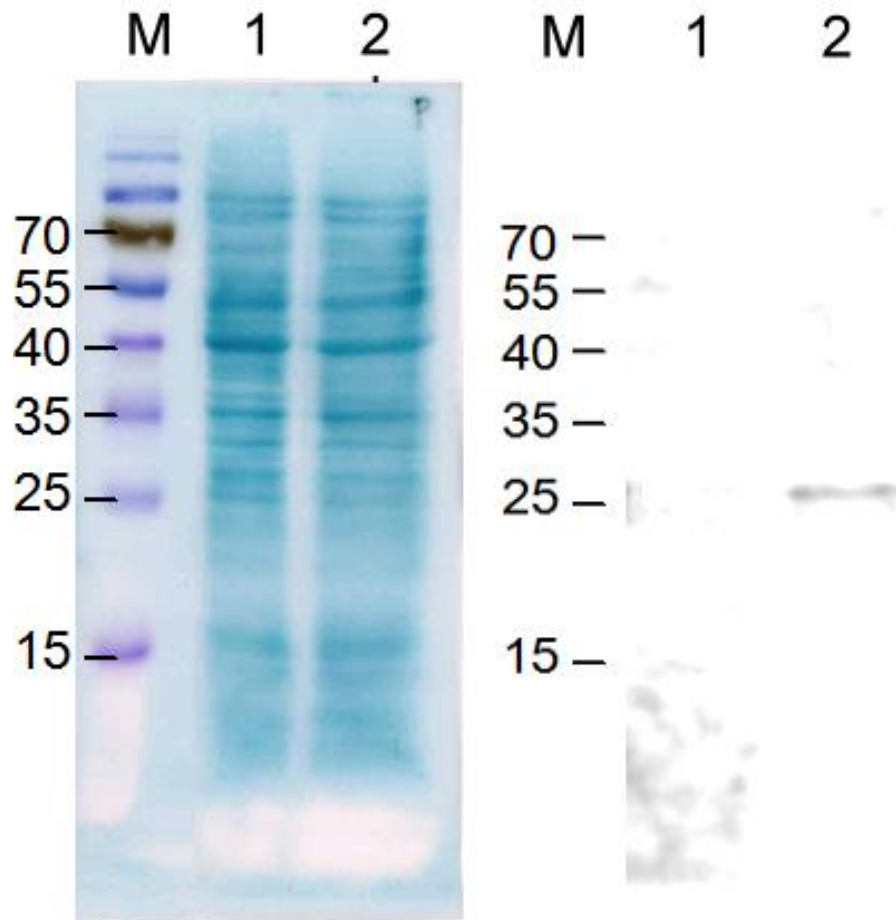
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Supplementary Figure 1: IgE antibodies to recombinant Tyr p 3 by ELISA. The IgE antibodies in serum samples from allergic patients who were sensitized to *T. putrescentiae* crude extract (5 µg/mL) were detected by ELISA. ELISA inhibition was performed by pre-absorbed with different allergens including *D. pteronyssinus*, *B. tropicalis* and rDer p 3. Absorbance of optimal density (OD) at 405nm. Positive response in the rectangle. ○ : healthy subjects (n=10); ●: allergic subjects without absorption (n=100); ▲: allergic subjects pre-absorbed with *D. pteronyssinus* (5 µg/mL)(n=100); ▲: allergic subjects pre-absorbed with *B. tropicalis* (5 µg/mL) (n=100); □: allergic subjects (n=100) pre-absorbed with Der p 3 (5 µg/mL). ○ Cut-off value: 0.25 (mean of normal subject's ± 2 standard derivations).



Supplementary Figure 2. (A) Protein profiles of crude extracts from *B. tropicalis* and *T. putrescentiae* were analyzed on 12% SDS-PAGE. M: Molecular marker; Lane 1: crude extracts of *B. tropicalis* (15 μ g); Lane 2: crude extracts of *T. putrescentiae* (15 μ g) (B) Western blot analysis with rabbit anti-Tyr p 3 polyclonal antibodies.

Tyrophagus putrescentiae clone CnTp-2 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: **GQ205624.1** Length: 487 Number of Matches: 1
Range 1: 1 to 487

Score	Expect	Identities	Gaps	Strand	Frame
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Query 1	CGACTTTTCGAACGCATATTGCAGCCGAGGTTATACCTCGGCTTCGTTTGTCTGAGCGTCG	60			
Sbjct 1	CGACTTTTCGAACGCATATTGCAGCCGAGGTTATACCTCGGCTTCGTTTGTCTGAGCGTCG	60			
Query 61	TTAATATGCCAAACACTATGCAAAGGCCCTGTTCCCATGTCGTGTCGATCATTCAAGTCAT	120			
Sbjct 61	TTAATATGCCAAACACTATGCAAAGGCCCTGTTCCCATGTCGTGTCGATCATTCAAGTCAT	120			
Query 121	CGACTCGTCAGGCCACTTGTAACTACTTTCAACTTTGTTATTGAACTGCCCATACGAG	180			
Sbjct 121	CGACTCGTCAGGCCACTTGTAACTACTTTCAACTTTGTTATTGAACTGCCCATACGAG	180			
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Sbjct 301	AGCACTGTGTGCGATCGATGCACCAAGCGCTTAAAGCTGTGCGCGTCTCCAATTACAATC	360			
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Sbjct 421	GCACATTTGTGAGTTGTTGTTAACTTTTCGACCTCAGATCAAGCGAGATTACCCCTGAA	480			
Query 481	TTTAAGC	487			
Sbjct 481	TTTAAGC	487			

Supplementary Figure 3: The contaminated specimen was selected for molecular identification, the sequence analysis of ITS-2 PCR product confirmed by Basic Local Alignment Search Tool (BLAST) on the website of NCBI. The partial sequence of ITS-2 was identical with *T. putrescentiae* partial sequence ID: GQ205624.1.