

## Original Article



# Survey of IgE Reactivity to Nonbiting Midges in Korea and Identification of IgE-Binding Protein

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There are no financial or other issues that might lead to conflict of interest.

## ABSTRACT

**Purpose:** Chironomids (nonbiting midges) are widely and abundantly distributed near ponds, rivers, and artificially dammed pools used for irrigation. Chironomids contain allergens and cause airway allergy in humans. In this study, we aimed to examine the allergic potential of chironomids in inhabitants living near artificially dammed pools.

**Methods:** We examined immunoglobulin E (IgE) reactivity to chironomid extracts in the sera of residents living around installed dams and assessed the correlations of IgE responses between chironomids (*Chironomus flaviplumus*, *Chironomus kiiensis*, *Cricotopus bicinctus*) and house dust mites (*Dermatophagoides farinae*). In addition, we identified potential IgE binding proteins specific for adult *C. bicinctus*, a popular species in Korea. Specific IgE antibodies in sera collected from the participants against the extracts were tested using enzyme-linked immunosorbent assay (ELISA).

**Results:** The average IgE-positive rates were 10.4%, 8.1%, and 8.2% in *C. bicinctus*, *C. flaviplumus*, and *C. kiiensis*, respectively. The IgE-positive rate and IgE titer of *C. bicinctus* antigen were higher in residents living around installed dams than in those who lived other places ( $P = 0.013$ ). Western blotting using sera having high IgE titers to *C. bicinctus* in ELISA showed the presence of a protein of approximately 42 kDa that was homologous to the actin protein isoform in *C. bicinctus* extracts as demonstrated using mass spectrometry.

**Conclusions:** Our results showed that people living near installed dams were more sensitized to *C. bicinctus* and that the 42 kDa IgE-binding protein could be useful for further studies on chironomid allergic disease and clinical applications.

**Keywords:** Nonbiting midges; IgE binding protein; allergy

## INTRODUCTION

Chironomids (nonbiting midges) inhabit natural rivers, lakes, and ponds as well as artificially dammed pools. Because adults have a short life span and weak bodies when debris from their bodies is mixed with dust or the air, this debris can then enter the nasal cavity or bronchus during breathing and cause allergies.<sup>1,2</sup> Hemoglobin are unique components of chironomid larvae and have been identified as potent allergens found in patients with allergies.<sup>3-6</sup> However, since hemoglobin are observed only in chironomid larvae, not adults,<sup>7,8</sup>

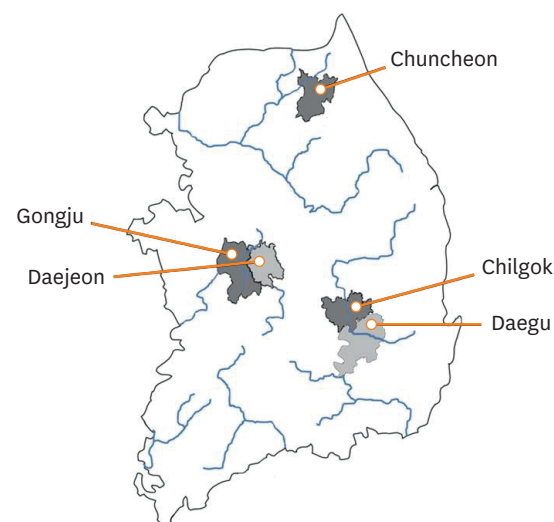
it is anticipated that other antigens from adult chironomids may induce allergic reactions such as asthma. When testing crude extracts of adult of chironomids (*Cricotopus sylvestris*), nearly 40% of sera from patients with allergic asthma were found to have *C. sylvestris*-specific immunoglobulin E (IgE) in Japan, Taiwan and Sweden.<sup>9</sup> Moreover, Kimura *et al.*<sup>10</sup> found that chironomid antigens are widely distributed in air, soil, and indoor dust near lakes in Japan. In Korea, two cases of asthma caused by *Chironomus plumosus* and *Tokunagayusurika akamusi* were reported in 1991.<sup>11,12</sup> The most common dominant species in Korea are *Chironomus flaviplumus* and *Chironomus kiiensis*, and both of these species are known to have allergens that can cause allergies.<sup>2,13</sup> Although *Chironomidae* is one of the most diverse and abundant groups of insects found in various habitats worldwide, it is still unclear whether they harbor allergens.

According to a report published by the Korea Water Resources Corporation in 2016,<sup>14</sup> the predominant species of chironomids in the major rivers in Korea after establishment of artificial dams are *C. flaviplumus*, *C. kiiensis*, and *Cricotopus bicinctus*. In the present study, we compared IgE levels specific for these three chironomids from residents living near artificially dammed pools by enzyme-linked immunosorbent assay (ELISA). Additionally, we investigated the association between specific IgE levels and the living area of the participants. Finally, we identified IgE-binding protein in whole-body extracts of *C. bicinctus* adults.

## MATERIALS AND METHODS

### Participants and serum samples

We obtained sera from subjects who came to a Medicheck branch of the Korea Association of Health Promotion for a health checkup from March to May 2016. We obtained sera from individuals in Gongju and Daejeon in Chungnam province and Chilgok and Daegu in Gyeongbuk province. We classified Gongju and Chilgok as areas around the installed dam (within 14 km of an installed dam), and Daejeon and Daegu as other areas (living more than 40 km away from a dam). In addition, we obtained sera from individuals in Chuncheon in Gangwon province, who are expected to be exposed to aquatic insects at a higher frequency than those living in the other two provinces, due to the presence of a large nearby lake (**Fig. 1**). More than 95% of the subjects



**Fig. 1.** The survey areas in Korea.

**Table 1.** Demographic and survey information on the study population

Characteristics	Chungnam		Gyeongbuk		Gangwon
	Gongju (n = 98)	Daejeon (n = 93)	Chilgok (n = 108)	Daegu (n = 112)	Cuncheon (n = 197)
Living around the dam	Yes	No	Yes	No	No
Age	67.8	59.4	68.3	65.5	48.7
Sex (M/F)	39/59 (40%/60%)	41/52 (44%/56%)	45/63 (41.7%/58.3%)	40/72 (35.7%/64.3%)	86/115 (43.7%/56.3%)

had lived in the area for more than 20 years. Information regarding the sera obtained from each region is shown in **Table 1**.

### Preparation of crude extracts

Chironomid adults and house dust mite (*Dermatophagoides farinae*) extract were purchased from the Arthropods of Medical Importance Resource Bank (Seoul, Korea).

### IgE reactivities of crude extracts

The reactivities of specific IgE antibodies to crude extracts were examined by ELISA. ELISA plates were coated with 100  $\mu$ L (0.1 M sodium carbonate, pH 9.6) of 2  $\mu$ g/mL crude extract, and plates were washed 3 times with phosphate-buffered saline containing 0.05% Tween 20 (PBST) and blocked with 3% skim milk in PBST for 1 hour. Subsequently, the plates were incubated with 100  $\mu$ L/well of sera diluted 1:4 in PBST containing 1% bovine serum albumin (BSA) for 1 hour. IgE antibodies were detected using biotinylated goat anti-human IgE (epsilon chain specific; Vector Laboratories, Burlingame, CA, USA) and streptavidin-peroxidase (Sigma, St. Louis, MO, USA). The signal was developed using 3,3',5,5'-tetramethylbenzidine. The mean absorbance plus 2 standard deviations of sera from 30 healthy controls was used as a cutoff value.

### Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Protein extracts were reduced by heating with 5  $\times$  SDS-PAGE loading buffer (T&I, Chuncheon, Korea), loaded onto wells of 10% Tris-glycine gels, and separated by SDS-PAGE. For immunoblotting, proteins were then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% BSA for 1 hour at room temperature, after which, the membranes were incubated with serum samples diluted 1:10 in 3% BSA overnight at 4°C. The membranes were then washed 3 times with TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) for 10 minutes each and incubated with horseradish peroxidase-conjugated goat anti-human IgE (Bethyl, Montgomery, TX, USA) diluted 1:10,000 in 3% BSA for 1 hour at room temperature. Next, the membranes were washed, and immunoreactive bands were detected with enhanced chemiluminescence reagents (Amersham Biosciences, Princeton, NJ, USA).

### Nano-liquid chromatography-mass spectrometry (LC-MS) analysis

IgE-reactive proteins were identified by nano-LC-MS analysis at Life Science Laboratories (Seoul, Korea). Briefly, lysates containing 50  $\mu$ g protein were denatured by 10% SDS, and in-gel samples were destained using 50% CH<sub>3</sub>CN (H<sub>2</sub>O) solvent for 15 minutes. We then added 1  $\mu$ g trypsin solution to the destained in-gel samples and incubated the samples at 37°C for 16 hours. Next, we added dithiothreitol (1 M), stored the samples at room temperature for 1 hour, performed alkylation by adding iodoacetamide (1 M), and stored the samples again at room temperature for 1 hour. Fifty percent CH<sub>3</sub>CN solvent was added to recover the hydrolyzed peptides through gel dehydration.

### Statistical analysis

Chi-square tests and linear by linear association tests were performed using SPSS Statistics version 23.0 (IBM Corp., Armonk, NY, USA). Results with *P* values of less than 0.05 were considered significant.

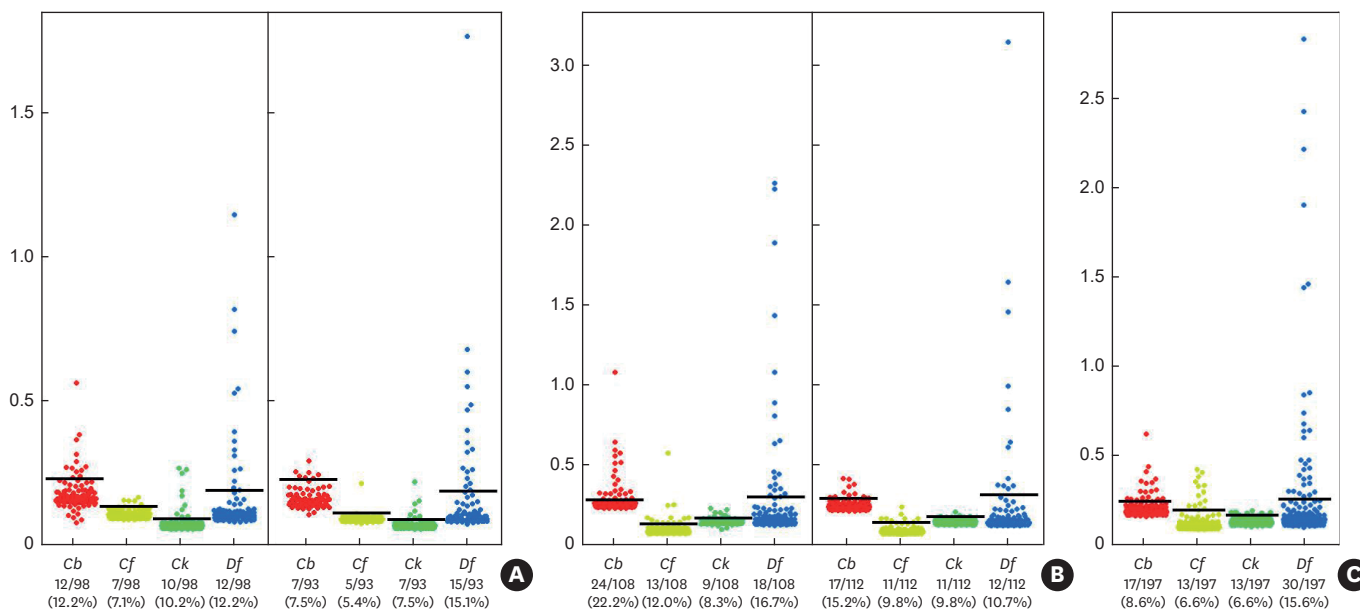
### Ethics statement

This study protocol was approved by the Institutional Review Board of Yonsei University Severance Hospital (approval No. 4-2015-0977), and each participant signed a written informed consent form.

## RESULTS

### IgE reactivity to *C. bicinctus*

The IgE-positivity rate and IgE levels specific for *C. bicinctus* extracts were higher in residents living around the installed dam (Gongju: 12.2%, 12/98) than in other residents (Daejeon: 7.5%, 7/93) in Chungnam (Fig. 2). In addition, the IgE levels and IgE reactivity *C. bicinctus* were higher in residents (22.2%, 24/108 persons) living around the installed dam (Chilgok) at another location (Daegu: 15.2%, 17/112 persons) in Gyeongbuk. Among residents in Gangwon, which is known to harbor abundant chironomids because of the presence of large lakes (Chuncheon) and rivers (Soyang), 8.6% (17/197) were positive for reactivity to *C. bicinctus*, and sIgE levels were also high (Fig. 2). These data demonstrated that the IgE-positivity rate and IgE reactivity against *C. bicinctus* extracts were higher in residents living around the installed dam than in those living in other places.



**Fig. 2.** The IgE reactivities to *Cb*, *Cf*, *Ck*, and *Df* using ELISA. (A) Sera collected from inhabitants living around the installed dam (left) and local controls (right) in Chungnam. (B) Sera collected from inhabitants living around the installed dam (left) and local controls (right) in Gyeongbuk (C) in Gangwon. The cut-off values (dashed lines) were set as the means plus two standard deviations for the control sera.

*Cb*, *Cricotopus bicinctus*; *Cf*, *Chironomus flaviplumus*; *Ck*, *Chironomus kiiensis*; *Df*, *Dermatophagoides farinae*; IgE, immunoglobulin E; ELISA, enzyme-linked immunosorbent assay.

### IgE reactivity to *C. flaviplumus* and *C. kiensis*

Because *C. flaviplumus* and *C. kiensis* are known to cause allergies, we confirmed IgE reactivity to these extracts in residents living around installed dams and those in other places by ELISA. The prevalence rates of IgE responses to *C. flaviplumus* extract were 7.1% (7/98) and 5.4% (5/93) in residents living around the installed dam (Gongju) and those living in another place (Daejeon) in Chungnam province, respectively (**Fig. 2A**). In Gyeongbuk province, the positivity rates of IgE responses to *C. flaviplumus* extract in residents living around the installed dam (Chilgok) and those in another area (Daegu) were 12.0% (13/108) and 9.8% (11/112), respectively. There were no differences in the residents between those 2 areas (**Fig. 2B**).

In Chungnam province, the IgE response rates of *C. kiensis* extracts were not different between residents living around the installed dam (10.2%, 10/98) and those living in other areas (7.5% 7/93). The IgE response values were also very low. In addition, the positivity rates for *C. kiensis* were 8.3% (9/108) and 9.8% (11/112), respectively, and there were no differences between residents living around the installed dam and those living in another place in Gyeongbuk province (**Fig. 2**). The IgE positivity rates of *C. flaviplumus* and *C. kiensis* extracts were 6.6% (13/197) and 6.6% (13/197) in Gangwon province (Chuncheon), which were similar to those in Chungnam province and Gyeongbuk province (**Fig. 2**).

### IgE reactivity to house dust mites

To compare the IgE reactivity to between chironomids and house dust mites, we used the house dust mite extracts, which are a major cause of allergic reactions. In Chungnam province, IgE reactivities to house dust mite extracts were 12.5% (12/98) and 16.1% (15/93) in residents of Gongju and Daejeon, respectively (**Fig. 2A**). In Gyeongbuk province, the positivity rates were 16.7% (18/108) in Chilgok and 10.7% (12/112) in Daegu. IgE reactivity to house dust mites did not differ between the residents living around the installed dam and those living in other places in either Chungnam province or Gyeongbuk province. The positivity rate of IgE to house dust mites in Gangwon province was 6.6% (30/197; **Fig. 2C**).

### Factors associated with IgE reactivity to *C. bicinctus*

Based on the results of ELISA, the IgE reactivities to *C. bicinctus* and house dust mites were analyzed in 608 participants in order to investigate the associations according to region, sex and age (**Table 2**). There were no differences among the 3 provinces in responses to *C. bicinctus* or house dust mites; however, those living around installed dams were found to have higher IgE responses to *C. bicinctus* than those living in other places ( $P = 0.013$ ; **Table 2**). Interestingly, IgE responses to *C. bicinctus* and house dust mites were found to be higher in males. In addition, the younger the subjects were, the more sensitized they were to house dust mites, whereas IgE reactivity to *C. bicinctus* not associated with the age of the participants. Interestingly, the results showed that there was a positive association between HDM-specific IgE reactivity and *C. bicinctus*-specific IgE ( $P < 0.001$ ; **Table 2**).

### Detection of IgE-binding antigens from *C. bicinctus*

*C. bicinctus* extracts separated by SDS-PAGE (**Supplementary Fig. S1**) were subjected to IgE immunoblotting with serum showing sensitization to *C. bicinctus* confirmed by ELISA. Interestingly, *C. bicinctus*-positive serum (**Fig. 3B**) reacted with a protein of approximately 42 kDa, whereas this band was not observed in *D. farinae*-positive serum.

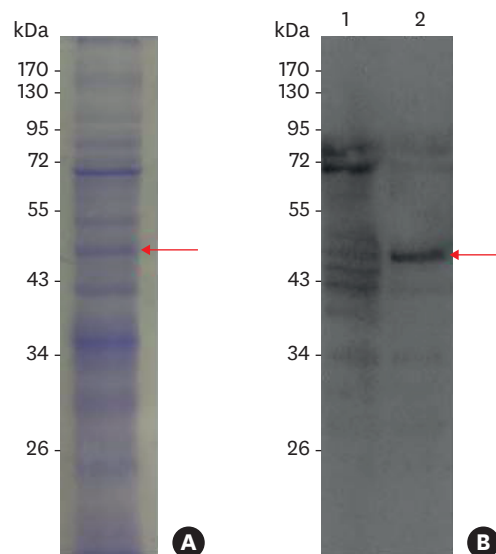
**Table 2.** IgE reactivities to *Cricotopus bicinctus* and *Dermatophagoides farinae*

Characteristics	IgE-reactive to <i>C. bicinctus</i>			IgE-reactive to HDM	
	Total	No. (%)	<i>P</i> value	No. (%)	<i>P</i> value
Region			0.464		0.895
Gangwon	197	17 (8.6)		30 (15.2)	
Chungnam	191	19 (9.9)		27 (14.1)	
Gyeongbuk	220	27 (12.3)		30 (13.6)	
Total	608	63 (10.4)		87 (14.3)	
Residents living around the installed dam*			<b>0.013</b>		0.683
Yes (Gongju, Chilgok)	206	31 (15.0)		30 (14.5)	
No (Daejeon, Daegu)	205	15 (7.3)		27 (13.2)	
Total	411	46 (11.2)		57 (13.8)	
Sex			<b>&lt; 0.001</b>		<b>&lt; 0.001</b>
Male	251	41 (16.3)		51 (20.3)	
Female	357	22 (6.2)		36 (10.1)	
Total	608	63 (10.4)		87 (14.3)	
Age			0.335		<b>0.006</b>
< 40	44	3 (6.8)		13 (29.5)	
40–49	84	8 (9.5)		17 (20.2)	
50–59	136	15 (11.0)		20 (14.7)	
60–69	172	13 (7.6)		20 (11.6)	
> 69	172	24 (14.0)		17 (9.9)	
Total	608	63 (10.4)		87 (14.3)	
HDM-specific IgE detection			<b>&lt; 0.001</b>	-	-
Positive	87 (14.3)	36 (57.1)			
Negative	521 (85.7)	27 (42.9)			
Total	608	63			

Bold styled values are presented as  $P < 0.05$ .

IgE, immunoglobulin E; HDM, house dust mite.

\*Yes: distance to dam < 14 km, No: distance to > 40 km.



**Fig. 3.** *Cricotopus bicinctus* extracts separated by SDS-PAGE (A) were subjected to immunoblotting with sera from individuals sensitized to house dust mite (lane 1) or *C. bicinctus* (lane 2) (B). SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

### Identification of IgE-binding proteins by nano-LC-MS analysis

The 42-kDa IgE-reactive component of the *C. bicinctus* extract was analyzed by nano-LC-MS after in-gel trypsin digestion. Peptide spectrum matching for the 42-kDa band showed the highest sequence coverage (63.03%) for CLUMA\_CG003758, isoform A (Table 3, with Score



**Table 3.** Identification of about 42-kDa IgE-binding protein from *Cricotopus bicinctus* extract

Accession No.	Putative identity	Coverage	Score	Sequest HT	MW (kDa)	Organism
CRK90033.1	Similar to Actin	63.03	143.15	41.65	41.65	<i>Clunio marinus</i>
CRK90035.1	Putative Actin	42.29	105.14	41.66	41.66	<i>Clunio marinus</i>
CRK87228.1	Similar to Actin	36.70	94.37	41.78	41.78	<i>Clunio marinus</i>
CRL01468.1	Putative phosphoglycerate kinase	30.12	67.92	43.92	43.92	<i>Clunio marinus</i>
CRK93344.1	Putative Enolase	48.97	258.77	47.25	47.25	<i>Clunio marinus</i>

IgE, immunoglobulin E; MW, molecular weight.

Sequest HT), which is similar to actin in *Clunio marinus* (National Center for Biotechnology Information [NCBI] accession No. CRK90033.1). **Table 3** summarizes the mass spectrometry analysis of the bands.

## DISCUSSION

Chironomids are found in almost all aquatic habitats in Korea, exhibiting a wide range of species diversity.<sup>2,15</sup> Although chironomids and their larvae are known to contain potent allergens, there has been little research on the allergic responses to chironomids in Korea. Recent studies have reported that environmental changes have caused the number of unpleasant insects, such as nonbiting midges, to increase around rivers. In 2013, a report by the Korean Water Resources Corporation showed that *Chironomidae* species were prominent in 4 major rivers in Korea.<sup>16,19</sup> Such abundance of chironomids increases the chance of contact with humans, leading to allergic disorders in susceptible individuals. Chironomids have been known as a major cause of allergic disease since the late 1970s. After the construction of a large dam on the Nile River in Sudan, the number of patients with allergic asthma increased due to an increase in the incidence of *Cladotanytarsus lewisi*.<sup>20,21</sup> Since then, a number of allergic diseases have been reported among aquarium and feed mill workers handling *Chironomus thummi* larvae, which are used as fish feed.<sup>22</sup> Kagen *et al.*<sup>23</sup> reported the occurrence of atopic disease caused by *C. plumosus* antigens in a reservoir in Wisconsin, USA. In Japan, asthmatic children in Toyama were positive for IgE reaction to *Polypedilum kyotoensis*, *Chironomus yoshimatsui*, and *T. akamusi*, and 57.1% of asthmatic patients in Kyoto were positive for IgE reaction to *C. yoshimatsui*.<sup>24,25</sup> In Korea, Park *et al.*<sup>11</sup> reported 2 patients with bronchial asthma who were positive for IgE reaction to *C. plumosus* and *T. akamusi* in 1991. In addition, Kim *et al.*<sup>12</sup> reported that among 475 asthmatic patients, 20% and 20.6% were positive for *C. plumosus* and *T. akamusi*, respectively. Asthmatic patients with positive responses to various species of chironomids have been reported. Since there can be immunological cross-reactions between species, it is possible that sensitization to one species induces allergic responses to other species.

In the present study, we examined sera having IgE reactions to crude extracts of *C. flaviplumus*, *C. kiiensis*, and *C. bicinctus*. Several studies have shown that the dominant species *C. flaviplumus* and *C. kiiensis* induce allergies; however, this is the first study showing that *C. bicinctus* can increase IgE levels and potentially cause allergic reactions. We also investigated whether *C. flaviplumus*, *C. kiiensis*, and *C. bicinctus* could increase IgE responses in residents living around the installed dam, where the emergence of chironomid is expected to be high.

In Chungnam and Gyeongbuk residents living around installed dams, the IgE response against *C. bicinctus* was significantly higher than in residents who did not live around dams. However, the specific IgE levels and positivity rates (%) of IgE reactivity to *C. flaviplumus* and *C. kiiensis* were not different between residents living around installed dams and those living

elsewhere in Chungnam and Gyeongbuk. These data showed that *C. bicinctus* might have higher allergenicity than *C. flaviplumus* and *C. kiensis* for individuals living around installed dams. Based on these results, we assumed that individuals living around installed dams would have a higher chance of being exposed to aquatic insects, such as chironomids, than those living in other places. Further investigations are needed to determine whether other species of *Chironomidae* could increase IgE.

In addition, we compared the IgE reactivity of house dust mites, a major allergen, with that of chironomid extracts. The results showed that the specific IgE levels against chironomid extracts in the sera of individuals living in Chungnam, Gyeongbuk, and Gangwon were lower than those against house dust mites in the same individuals. Moreover, the IgE response rates to house dust mites were more than 10% in all individuals in the 3 provinces, suggesting a high prevalence.

Investigating immunological cross-reactions is important, both clinically and experimentally.<sup>26,27</sup> Some studies have reported on cross-reactivity between house dust mites and nonbiting midges, although there is still controversy over whether they cross-react. In 1989, Yamashita *et al.*<sup>28</sup> found no significant correlations between positive skin test results with *Chironomidae* and those with other allergens (house dust mite, silk, shrimp, or mosquito allergens). In contrast, another study claimed that a chironomid midge (*C. yoshimatsui*) positive for IgE reaction exhibited cross-reactivity with a house dust mite (*Dermatophagoides pteronyssinus*).<sup>29</sup> A study by Witteman *et al.*<sup>30</sup> showed that patients sensitized to house dust mites had cross-reactivity to silverfish, cockroaches, and chironomids. Pascual *et al.*<sup>31</sup> found an IgE-binding protein of 30-43 kDa to which *Anisakis* nematodes, German cockroaches, and chironomids responded in common. In this study, individuals who were positive for IgE reactivity to house dust mites also tended to respond to *C. bicinctus*; however, we did not find any common IgE-binding antigens. Our result showed that IgE responses to house dust mite extracts were correlated with IgE responses to *C. bicinctus*, although the IgE-binding antigens of *C. bicinctus* might be different from those of house dust mites. We assumed that some individuals may be more susceptible to allergens, resulting in immune responses that elevate IgE to both chironomids and house dust mites.

Several lines of evidence have indicated the existence of IgE cross-reactivity among chironomid species and identified common antigenic determinants. In 1983, it was reported that 14 species of chironomids exhibited cross-reactivity and that hemoglobin in the larval stage affected these cross-reactions.<sup>32</sup> Tee *et al.*<sup>33</sup> reported cross-reactivity between *C. lewisi* and *Chironomus riparius*. In Korea, cross-reactivity between *C. plumosus* and *T. akamusi* has been confirmed in asthmatic patients by competitive inhibition ELISA.<sup>12</sup> Unfortunately, in this study, we were unable to investigate the cross-reactivity of the three species of chironomids tested; we cannot exclude the possibility that there is cross-reactivity among them.

Various arthropod allergens, such as tropomyosins, myosins, paramyosins, glutathione S-transferase, arginine kinase, and fatty acid binding proteins ([www.allergome.org](http://www.allergome.org)) have been identified. Previous studies have shown that tropomyosin is an important adult chironomid allergen.<sup>34</sup> Notably, tropomyosin contains a group of highly conserved actin-binding proteins and plays a central role in muscle contraction.<sup>35</sup> Several studies have shown that tropomyosin is an invertebrate pan-allergen because of its high cross-reactivity in sensitized individuals with shellfish or house dust allergies.<sup>34-36</sup> In our study, we detected a 42-kDa human IgE-binding antigen in *C. bicinctus* extracts, which was confirmed as actin.



Actin is the most abundant protein in eukaryotic cells,<sup>37</sup> although its ability to induce allergic reactions has not been extensively studied. Recent studies have shown that actin from clams plays a pivotal role in patients with local allergy.<sup>38</sup> Although no studies have demonstrated that insect-derived actin induces allergies, in this study, our findings suggested that people living around installed dams develop IgE reactivity from actin derived from chironomids. However, further studies are needed to investigate whether IgE responses in serum are due to cross-reactivity between chironomid-derived actin and other allergens.

There were some limitations to this study. For example, we did not consider whether individuals had allergic symptoms or a history of allergy during recruitment. Instead, we focused on whether increased chironomid populations caused by artificial environment changes, such as the construction of dam, could increase the number of individuals with IgE reactivity. Accordingly, additional studies are needed to evaluate the effects of chironomids on allergic symptoms and allergy. In this study, IgE-reactive actin was identified in the serum with the highest IgE titer as measured by ELISA. In the other cases in which the IgE titer was lower, this protein band was not detected. We therefore cannot conclude that this protein is a major allergen. However, we speculate that the subject with the highest specific IgE titer has a greater probability of being sensitized to this protein.

In conclusion, our results showed that more people living around installed dams were sensitized to *C. bicinctus* than those living in other places. In addition, we also investigated actin from chironomids as a novel IgE-responsive antigen and found that the detected antigen may trigger allergic responses. Further studies are needed to investigate whether chironomids may cause allergies.

## ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

### Supplementary Fig. S1

(A) Features of *Cricotopus bicinctus* adults on stereoscopic microscopy. (B) SDS-PAGE of crude extracts from *Chironomus flaviplumus* (lane 1), *Chironomus kiensis* (lane 2), and *C. bicinctus* (lane 3).

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