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Original Article

Allergenic importance of 22 species of Japanese chironomid midges

Koichiro Kawai,[1] Gyokei Murakami,[2] Shiro Kasuya,[3] Hidetoyo Teranishi[4] and Atsushi Muraguchi[5]

[1]Laboratory of Ecology, Faculty of Applied Biological Science, Hiroshima University, Hiroshima, [2]Department of Pediatrics, Toyama Red Cross Hospital, Toyama, [3]Department of Parasitology, School of Medicine, Gifu University, Gifu, and Departments of [4]Public Health and [5]Immunology, School of Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan

ABSTRACT

Twenty-two Japanese chironomid species were examined for their allergenicities using ELISA with the sera of 32 asthmatic patients. The species *Paratrichocladius rufiventris* and *Cricotopus sylvestris* showed high positive rates of specific IgE, high average IgE reactivities and high frequencies of strong IgE reactivity and the highest IgE reactivity of the 22 species, suggesting a high rate of contact with humans and the possession of highly allergenic components by these two species. In contrast, *Tanypus punctipennis* and *Rheotanytarsus kyotoensis* showed low allergenicities, suggesting a low level of human contact and/or a lack of allergenic components. Furthermore, species that emerge from eutrophic waters in a large mass, such as *Macropelopia paranebulosa*, *Paratrichocladius rufiventris* and *Chironomus yoshimatsui*, showed strong allergenicities in all the tests. This suggests that eutrophic water be regarded as an important reservoir to allergenic chironomids.

Key words: allergen, chironomid, cross-allergenicity, ELISA.

Corespondence: Dr Koichiro Kawai, Laboratory of Ecology, Faculty of Applied Biological Science, Hiroshima University, 1-4-4 Kagamiyama, Higashihiroshima-shi, Hiroshima 739, Japan.

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INTRODUCTION

A dipteran insect family Chironomidae has been proved worldwide to be a potent human allergen.[1–4] However, of the several thousands of species recorded worldwide, only 38 species belonging to 19 genera have been examined for allergenicities using prick tests and radioallergosorbent test (RAST).[5–11] Cranston *et al.* examined the allergenicities of eight Nilotic chironomid species, including *Cladotanytarsus lewisi*, a midge that emerge from the River Nile Basin in an enormous mass.[7] They used skin prick tests in Sudanese and Egyptian asthmatic subjects, and demonstrated that about 80% of asthmatics showed positive reactions to *C. lewisi* and that more than 40% of *C. lewisi*-positive subjects showed positive reactions to the species of the genera *Dicrotendipes*, *Procladius* and *Conchapelopia*, while less than 20% of *C. lewisi*-positive subjects showed positive reactions to genera *Paracladopelma*, *Nanocladius*, and *Cryptochironomus*.[7] Baur *et al.* also examined the allergenicities of 25 species of different taxa using RAST and the sera of German and American asthmatics, thought to be sensitized by *Chironomus thummi thummi* and *C. plumosus*, respectively, and demonstrated that evolutionarily closely related species, such as species of the genera *Chironomus*, *Glyptotendipes* and *Polypedilum*, showed moderate to strong binding to the patients' IgE.[6]

In the present study, 22 species of different genera, distributed widely in various types of waters in Japan, including 11 genera that had not been previously examined for allergenicities, were examined for their reactivity with IgE in asthmatic patients' sera using ELISA.

METHODS

Chironomid midges

Twenty-two species, each belonging to a different genus, of three chironomid subfamilies were used (Table 1). Among these, midges of CS, CaB, ChY, GT, PeS, PoN, PtG and TaO species were cultured in the laboratory. ToA midges were obtained by rearing the larvae purchased from a commercial source in Osaka, Japan. Midges of other species were obtained by rearing the larvae hatched from egg masses collected in the field. The evolutionary relationships between the 21 genera, using the systems of Fittkau[24] and Saether,[25,26] are shown in Fig. 1.

Patients

Sera of 32 asthmatics (range, 6–69 years old, mean, 25.1; 25 males, 7 females) were obtained from the Department of Internal Medicine of Tohno Hospital, from the Departments of First Internal Medicine and Pediatrics of Toyama Medical and Pharmaceutical University Hospital and from the Department of Pediatrics of Toyama Red Cross Hospital. All the patients had recurrent acute attacks of wheezing, dyspnea, cough and expectoration of mucoid sputum and showed positive IgE reactions to at least one species of chironomid in RAST, RAST enzyme immunoassay or ELISA tests.

Midge extracts

Midge extracts were prepared by the methods described previously.[27] Briefly, triturated midges were defatted with ether and extraction was performed with phosphate-buffered saline (PBS), pH 7.5, for 48 h at 4°C, with gentle stirring. After centrifugation, the supernatant was dialysed against PBS using Spectrapor 3 tubing (Spectrum Medical Industries, USA; MW exclusion 3500 Da). The material retained in the tubing was centrifuged, and the supernatant was used as the crude extract.

ELISA

ELISA was performed using 96-well microtiter plates. After coating each well with midge crude extract at 10 μgP/mL in a coating buffer containing 0.1 mol/L $NaHCO_3$ and 0.02% NaN_3, pH 9.6, overnight at 4°C,

Table 1. The 22 chironomid species studied and their major habitats

Species	Abbrev.	Habitats
Tanypodinae		
Ablabesmyia monilis	AM	Oligo- to mesotrophic rivers and lakes[12]
Macropelopia paranebulosa	MP	Rice paddies[13]
Rheopelopia maculipennis	RM	Meso- to eutrophic rivers[14]
Tanypus punctipennis	TP	Rice paddies[13]
Orthocladiinae		
Cricotopus sylvestris	CS	Eutrophic lakes[15,16]
Hydrobaenus biwaquartus	HB	Eutrophic lakes[15]
Paratrichocladius rufiventris	PR	Eutrophic rivers and lakes[16]
Psectrocladius aquatronus	PsA	Oligo- to mesotrophic rivers and lakes[12]
Tokunagayusurika akamusi	ToA	Meso- to hypertrophic lakes[7]
Chironominae		
Chironomini		
Camptochironomus biwaprimus	CaB	Eutrophic lakes[15]
Chironomus yoshimatsui	ChY	Eutrophic rivers[18]
Dicrotendipes pelochloris	DP	Eutrophic lakes [19]
Glyptotendipes tokunagai	GT	Eutrophic lakes[19]
Microtendipes pedellus	MiP	Oligo- to mesotrophic rivers and lakes[12]
Parachironomus digitalis	PaD	Oligotrophic rivers[20]
Paratendipes tamayubai	PdT	Oligotrophic rivers[21]
Pentapedilum sordens	PeS	Meso- to eutrophic lakes[22]
Polypedilum nubifer	PoN	Eutrophic lakes[19] and rivers[21]
Stictochironomus multannulatus	SM	Oligo- to mesotrophic rivers and lakes[12]
Tanytarsini		
Paratanytarsus grimmii	PtG	Lakes, ponds, reservoirs, aquaria, water distribution systems[23]
Rheotanytarsus kyotoensis	RtK	Eutrophic rivers[18]
Tanytarsus oyamai	TaO	Oligo- to eutrophic rivers[12] and eutrophic lakes[19]

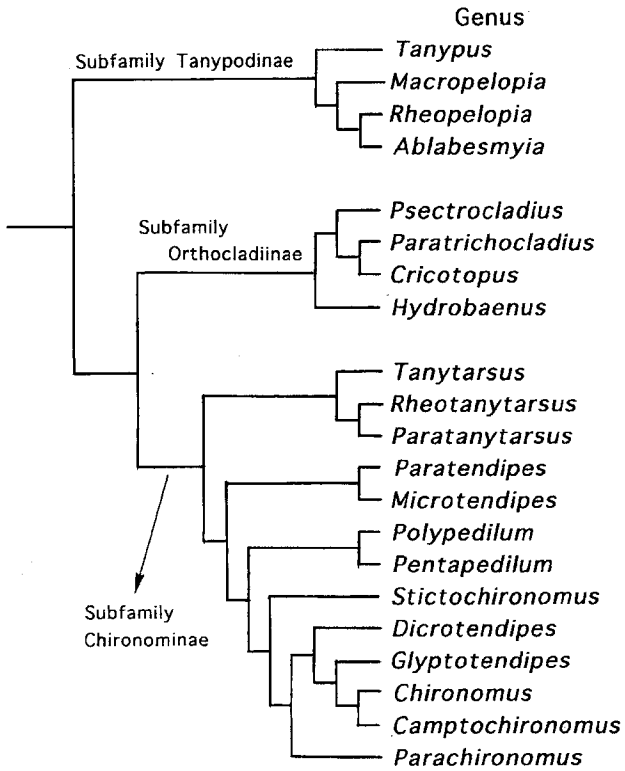

Fig. 1 Evolutionary relationships between 21 genera of Chironomid midges. The genus *Tokunagayusurika* was excluded due to no information on its evolution.

the wells were washed and filled with patients' sera at 1/10 dilution with diluent buffer containing 0.05 mol/L Tris, 0.001 mol/L $MgCl_2$, 0.15 mol/L NaCl, 0.05% Tween20, 0.02% NaN_3 and 1% bovine serum albumin, and incubated for 2 h at room temperature. After washing, the wells were filled with HRP-conjugated goat anti-human ϵ-chain IgG antibody (DAKO, Glostrup, Denmark) at 1/2000 dilution and incubated for 2 h at room temperature. After washing, a substrate solution containing 2.5 g of o-phenylenediamine and 500 μL of hydrogen peroxide per liter of 0.033 mol/L citrate and 0.066 mol/L Na_2HPO_4 was pipetted in, and the reaction was stopped with 2 N sulfuric acid. Absorbance at 492 nm subtracted by that at 405 nm, both measured by an ELISA reader (Bichromatic-348, Labsystems Multiskan, Helsinki, Finland), were represented as the ELISA values.

RESULTS

IgE reactivity of a pooled cord serum

A pooled cord serum from eight infants was examined for IgE reactivity with extracts of the 22 species (Table 2).

Table 2. IgE reactivity of a pooled cord serum

Species	ELISA value
AM	0.05
MP	0.04
RM	0.04
TP	0.04
CS	0.04
HB	0.10
PR	0.04
PsA	0.04
ToA	0.05
CaB	0.09
ChY	0.13
DP	0.13
GT	0.08
MiP	0.12
PaD	0.04
PdT	0.08
PeS	0.13
PoN	0.04
SM	0.04
PtG	0.04
RtK	0.08
TaO	0.04

Quite high IgE reactivities were shown to HB, CaB, ChY, DP, GT, MiP, PdA, PeS and PtK. Low values were observed for the other 13 species.

Positive rates of specific IgE in patients' sera (Fig. 2)

When the ELISA value of a patient's serum to a species was higher than twice that of the pooled cord serum to that same species, it was regarded as positive IgE reactivity, and positive rates were compared among the species. High positive rates were observed to almost all the species belonging to the subfamilies Tanypodinae and Orthocladiinae. In contrast, that positive rate differed from species to species for Chironominae. Of the species of Chironominae, the highest positive rate was shown to PoN. Positive rates higher than 50% were also shown to ChY, PaD, SM, PtG and TaO. Only low positive rates were observed for DP, PdT and PeS.

IgE reactivities of the patients' sera (Fig. 3)

For each species, the ELISA value in a patient, obtained by subtracting the ELISA value for that species in the pooled cord serum, was regarded as the specific IgE reactivity, and specific reactivities were compared among

the species. The highest average reactivity was shown to PR. High average reactivities were also observed for AM, MP, CS and ChY. Average reactivity differed from species to species for subfamilies Tanypodinae and Orthocladiinae and tribe Chironomini. In contrast, weak reactivities were observed for all the species of tribe Tanytarsini.

Extremely strong IgE reactivity (i.e. specific ELISA values higher than 1.0) was shown to PR at the highest frequency of 5. The species AM, MP and MiP also showed strong reactivities at the high frequency of 3; CS, CaB, ChY, DP and SM also showed strong reactivities, although at low frequencies. Specific ELISA values higher than 1.0 were never observed for the other 13 species.

Frequencies of the highest ELISA value in each patient for the 22 species (Fig. 4)

The frequency of the highest ELISA value was examined in each patient for each of the 22 species, and PR showed by far the highest frequency of 9. CS, ToA, ChY and PoN also showed high frequencies of 4–5. AM, HB, PsA, GT and MiP also showed high values, although at low frequencies. The other 12 species never showed high values in any patient.

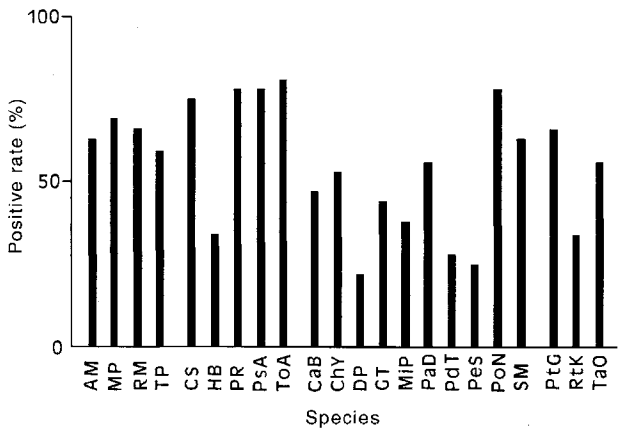

Fig. 2 Positive rates of specific IgE reactive to crude extracts of 22 species for 32 patients as assayed by ELISA. When the ELISA value of a patient's serum to a species was higher than twice that for a pooled cord serum it was regarded as positive. (See Table 1 for key to abbreviations.)

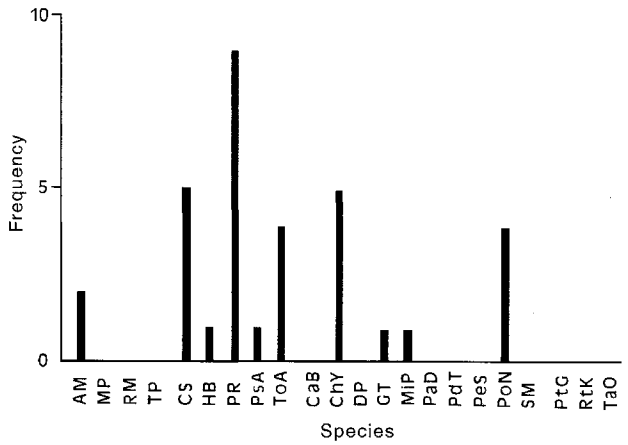

Fig. 4 Frequencies of the highest specific ELISA value in each patient for 22 species.

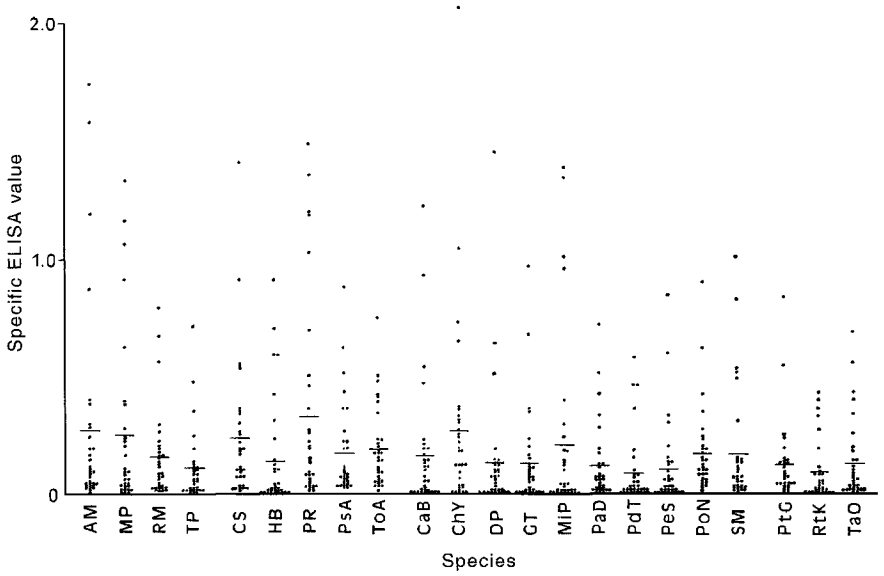

Fig. 3 IgE reactivity of patients' sera to crude extracts of the 22 species. The ELISA value of a patient's serum to a species subtracted by that for a pooled cord serum was regarded as the specific ELISA value. A horizontal bar represents the mean specific value of 32 patients. (See Table 1 for key to abbreviations.)

Discussion

In the study reported here, a total of 22 species of chironomid midges, each belonging to a different genus, were examined by ELISA for allergenicities in humans from different aspects; that is, the positive rate of specific IgE, the average IgE reactivity, the frequency of strong reactivity and the frequency of the highest reactivity. All the genera except for *Paratendipes*, *Pentapedilum* and *Rheotanytarsus* were shown to be allergenic at least in one aspect. Seven genera, *Ablabesmyia*, *Rheopelopia*, *Tanypus*, *Hydrobaenus*, *Paratrichocladius*, *Psectrocladius* and *Stictochironomus*, were first shown to be allergenic by this study.

The importance of chironomid species as an allergen needs to be considered from two different viewpoints; that is, the probability of sensitization and the severity of the allergic reaction. The former may be reflected by the positive rate of specific IgE and average specific IgE reactivity, while the latter may be reflected by the frequencies of strong IgE reactivity and the highest IgE reactivity in a patient.

The species PR and CS may have many opportunities for contact with humans and/or may contain a large amount of highly allergenic materials as these two species showed high positive rates of specific IgE, high average IgE reactivity and high frequencies of strong reactivity and the highest reactivity (Figs 2–4). Indeed, PR has been reported to emerge in considerably large numbers from an agricultural canal in Toyama City.[28] In contrast, TP and RtK may have low opportunities for contact and/or may contain little or no allergenic materials as no allergenicities were detected in any of the aspects examined. It is most likely that these species lack allergenic components because they also emerge in considerably large numbers from agricultural canals or rice fields, at least in Toyama City.[28,29] On the other hand, CaB and DP may have less opportunity for contact but may contain a considerable amount of allergenic materials to explain the low specific IgE positive rates and strong IgE reactivities in some patients. Conversely, RM may have many opportunities for contact but may contain only low amounts of allergenic materials, as this species showed a relatively high specific IgE positive rate but did not show strong IgE reactivity or the highest reactivity in any patient.

In the present study, PR and CS were proved to have strong allergenicities for humans in all the aspects examined (Figs 2–4). The result has profound implications because these species emerge in large numbers from various highly eutrophicated waters[14–16,28] located in the Japanese metropolitan areas. MP, ChY and PoN also showed high allergenicities and these species also emerge from eutrophicated waters.[13,16,18,21] Therefore, eutrophicated water should be regarded as a reservoir for air-borne allergenic chironomid midges.

High specific IgE positive rates were shown with almost all the species of the subfamilies Tanypodinae and Orthocladiinae (Fig. 2). This is possibly due to strong cross-allergenicities among these species attributable to chitin (β-poly-*N*-acetyl D-glucosamine), which is found in all arthropods,[30] and possessed by these subfamilies in large amounts. This hypothesis is supported by the fact that chitin exists as glycoproteins *in vivo*. Gad El Rab *et al.* have also reported that a major allergen of an African chironomid species, *Cladotanytarsus lewisi*, is an acidic glycoprotein.[31] However, the qualitative and quantitative differences of chitins need to be examined among chironomid species, or between chironomids and other arthropods such as mites and caddis flies.

In contrast, the specific IgE positive rate was quite different from species to species for the subfamily Chironominae, which is generally only weakly chitinized on any part of the body and usually has a large amount of hemaglobin (Hb) at the larval stage (Fig. 2). This suggests that neither chitin nor Hbs is mainly involved in the allergenicity of this subfamily. Judging by a report by Kawai and Sakamoto, murine IgE antibodies to some of the many Hb components possessed by a chironomid species more or less strongly cross-react even with Hbs of an evolutionarily distantly related species.[27] Indeed, many species, which do have Hbs at the larval and pupal stages, have been reported to lack detectable Hbs at the adult stage.[32,33] As well, Matsuoka *et al.* have shown no inhibition of specific IgE binding by larval extract to the extract of adult *Chironomus yoshimatsui*, suggesting the lack of involvement by Hbs in the allergenicity of adults.[33] However, they have also shown strong inhibition of specific IgE binding by egg extract to extract of adult *Tokunagayusurika akamusi*, suggesting the importance of vitellogenins as an allergen.[33] Therefore, chironomid allergens, including vitellogenins, other than chitin and Hbs should also be characterized and examined for cross-allergenicities.

In the present study, ChY showed a relatively high specific IgE positive rate while DP showed only a low positive rate, despite a close evolutionary relationship between these species (Figs 1,2). Similarly, PoN and PtG showed high positive rates while PeS and RtK showed only low

positive rates. Baur *et al.* also demonstrated strong IgE reactivities of the species of genus *Chironomus* in contrast to weak reactivity of the closely related genus *Dictrotendipes* using RAST with patients' sera that had been sensitized by *Chironomus* species.[6] On the other hand, Tee *et al.* reported the cross-allergenicity between *Chironomus riparius* of the tribe Chironomini of the subfamily Chironominae and *Cladotanytarsus lewisi* of another tribe of Tanytarsini of the same subfamily using RAST inhibition.[34] Kampen *et al.* also reported cross-allergenicity, even between *Chironomus* and *Cricotopus*, each belonging to a different subfamily, using RAST inhibition.[35] These results suggest that a similarity of composition of the allergenic components between the chironomid species does not necessarily reflect their evolutionary relationship. That is, the allergenic materials possessed by a species could be determined more by the environment or the life style of the species than by evolutionary factors. Therefore, the differences between chironomid species should be investigated at the individual allergen molecule level.

The population of asthmatic patients used in our study was considered to be heterogeneous with respect to sensitizing species or species composition. Therefore, the species composition of chironomid midges in the environment of each patient and, more exactly, the amounts of species-specific allergens should be investigated. Otherwise, comparison of the IgE-inducing potencies of different species using animal models of asthma is necessary in order to correctly determine the relative allergenicities of chironomid species.

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