論　文

PCR-RAPD 法によるケバエ科 2 種の遺伝的距離の測定

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Genetic distance in two Bibionid species by PCR-RAPD

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Abstract

In *Bibio rufiventris* (Dedu), 26 individuals from Kawajima and 19 from Oppe river (Takasaka), and, in *Bibio tenebrosus* (Conquillett), 3 from Kawajima, 8 from Oppe river and 74 from Daito Bunka University (the Higashi-Matuyama campus, Takasaka) were genetically analyzed by means of electrophoresis of random amplified polymorphic DNA, using one kind of RAPD-primer.

Bibio rufiventris and *tenebrosus* had the specific DNA markers clearly different from each other in the electrophoretic migration. Those are effective in classifying the two species. The genetic distance between the two species exceeded two times of the distance between any two local populations in each of the *rufiventris* and the *tenebrosus*.

要　旨

川島町と越辺川（高坂）から採集された、それぞれ 26 匹、19 匹のメスアカケバエ（*Bibio rufiventris*）と川島町、越辺川（高坂）、大東文化大学東松山キャンパスから採集された、それぞれ 3 匹、8 匹、74 匹のハグロケバエ（*Bibio tenebrosus*）について、1 種類の RAPD プライマーを使ってランダムに増幅された DNA 分子の多型を電気泳動法で調べた。

メスアカケバエとハグロケバエでは、それぞれ移動度の異なる種特異的な DNA マーカーを発現した。これらの DNA マーカーは 2 種類の分類に有用である。2 種類のケバエの遺伝的距離は、メスアカケバエとハグロケバエそれぞれのどの地域集団間の遺伝的距離と比べ、2 倍以上の大きさであった。

Introduction

Sympatric two Bibionid species of *Bibio rufiventris* (Duda) and *Bibio tenebrosus* (Coquillett) are clearly classified in the female. The former female has the pale-reddish-brown pronotum and abdomen and the latter female has black ones. On the other hand, males of the two species are not classified easily because those are black all over and the same in their morphology (Yasumatsu, Asahina and Ishihara, 1956). In microscopical comparison, the morphological difference was found. The *rufiventris* and the *tenebrosus* males have long and short bristles, respectively, on the upper side of head or the compound eyes. In their femeles, the similar difference was found, that is, the *rufiventris* and the *tenebrosus* femeles were long and short bristles, respectively, on the under side of the head (Hashimoto and Terata, 2018).

The present investigation is related to the genetic distances between the above two species and among local populations of the two species.

Material and Method

Collection of Bibionid species

In *Bibio rufiventris*, 26 individuals from Kawajima (the circumference of Kawajima agriculture co-operative store, Kawajima Town) and 19 from Oppe river (Takasaka, Higashi-Matsuyama City), and, in *Bibio tenebrosus*, 3 individuals from Kawajima(the same place as the above), 8 from Oppe river (Takasaka) and 74 from Daito Bunka University (the Higashi-Matuyama campus, Higashi-Matsuyama City) were collected in 2016. Those all flies were stored in the freezer, −80℃ .

Preparation of genomic DNA and DNA amplication

DNA was extracted from freezed legs by using the nucleic acid extraction kit: Kaneka Easy DNA Extraction Kit version 2, Kaneka Corporation.

Reactions were in volumes on 28 μl containing Ready-To-Go RAPD Analysis Beans: d.NTP mixture, PCR buffer and AmplyTaq DNA polymerase (GE Healthcare Limited), 2 μl of extracted genomic DNA solution containing 25 pmol DNA, and sterile distilled water. The amplification primer #1:5'-GGTGCGGGAA-3'.

PCR amplification was performed in the thermal cycler, AB Veriti Thermal Cycler, Applied Biosysytems, programed for 4 minutes at 95 ℃ followed by 45 cycles of 1 min., 95℃ , 1 min., 36℃ , and 2 min., 72℃ . The PCR products were automatically stored at 4℃ in the cycler.

Amplified DNA analysis

The amplified DNA was electrophoresed by Agilent 2100 Bioanalyzer Systems and DNA 7500 LabChip Kit (Agilent Technologies).

Bands or amplified DNA fragments were classified into 53 types: a to z, aa to zz and aaa, according to the degree of migration, in Figure 1 and Table 1 to 3. The band-appearance per individual and that per population were expressed as a percentage in every local population of the two species (figure 1 and 2). Genetic distances in the local population and the species were represented by the total and average amount of difference in appearance percentage of every locus or DNA-band expressible region. The average amount of difference per population or species was the total percent of difference divided by the number of compared populations or species. The genetic distance between the two species was calculated on the basis of the total of differences of all populations of every species.

Result and Discussion

Genetic markers of *Bibio rufiventris* and *Bibio tenebrosus*

Each of the *rufiventris* and the *tenebrosus* species had an unique band which is common with all individuals of each species experimented. The bands were different from each other in the electrophoretic migration. The remarkably blackish band of the *rufiventris* species was between 300 and 500 base pairs in DNA molecular size (R-mark in Figure 1 and gg-band in Table 1) and the relatively deep-grey band of the *tenebrosus* was near 700 base pairs (F-mark in Figure 1, s-band in Table 1). Those bands are probably genetic markers of the two species and are need to classify Bibionid species.

Genetic distance

In *Bibio rufiventris*, the total and average amounts of difference between Kawajima and Oppe river (Takasaka) were 38.78 and 19.39 persents, respectively. In *Bibio tenebrosus*, the total and average amounts between Kawajima and Oppe river , between Daito Bunka University (Takasaka) and Kawajima and between Daito Bunka University and Oppe river were 46.59 and 23.29, 52.26 and 26.13 and 49.92 and 24.96 percents, respectively (Table 4). All total percentages of differences in the *tenebrosus* species were larger than the total in the *rufiventris*. That seems to connect with the number of individuals experimented. Although the individuals used of Kawajima and oppe river were three and eight, those numbers were very small in comparison to the number of individuals, 74, of the Daito-Bunka-University population. Kawajima and Oppe river had the regions of DNA band or loci of 48.8 and 61.0 percents, respectively, of those of the Daito-Bunka-University. On the other hand, the commonality of locus in the two populations of the *rufiventris* species was 76.5 percent. Such a difference in the commonality seemed to generate the relatively large difference between the genetic distances of the *tenebrosus* group of population and the *rufiventris* one.

The total and the average differences between the *rufiventris* and the *tenebrosus* species were 120.24 and 60.12 percents, respectively (Table 3 and 4). Those total and average of differences are more than two times of the respective ones in the Bibionid population. Thus, the interspecific genetic distance

between *Bibio rufiventris* and *tenebrosus* are, probably, about twice as much as the intraspecific one in our experimental conditions. For obtaining accurate genetic-distance, examinations using many specimens and many kinds of DNA-primers or by means of DNA base sequence determination are necessary.

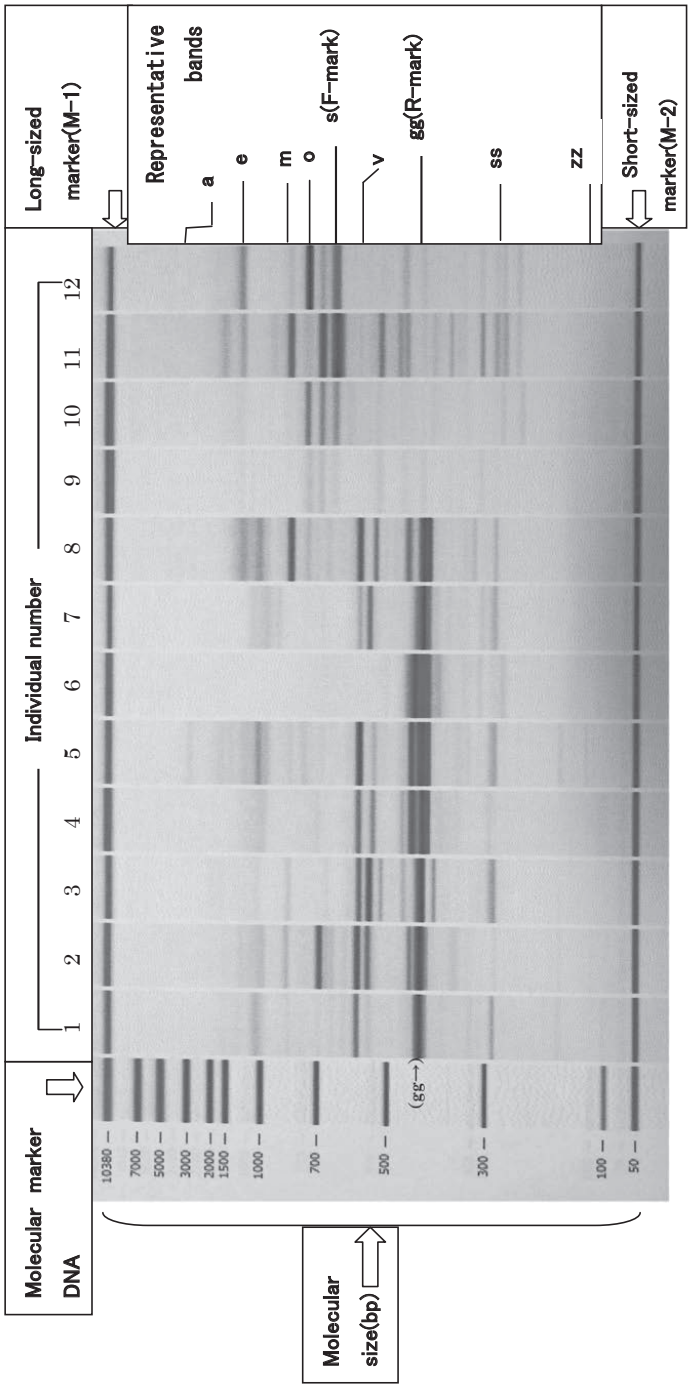

Figure 1. RAPD amplification patterns of genomic DNA from 12 Bibionid indivisuals of two *rufiventris* populations (Kawajima: number 1 to 4 and Oppe river: 5 to 8) and one *tenebrosus* population (Daito Bunka University: number 9 to 12). (F-mark and R-mark are the genetic marker bands of the *tenebrosus* and the *rufiventris* species, respectively.)

Table 1. Percentages of primer-1 bands in the *rufiventris* Kawajima and Oppe-river (Takasaka) populations (M-1and M-2 express long-sized and short-sized markers, respectively; symbols of a to z, aa to zz and aaa are DNA bands).

Collection place ↓	Appearance ↓	Bands→ (M-1)	a	b	c	d	e	f	g	h	i	j	k	l	m	n
Kawajima	per individual(%)→		7.69	7.69	7.69	0	30.77	11.54	23.08	57.69	3.85	3.85	15.38	0	61.54	11.54
	per population(%)→		0.64	0.64	0.64	0	2.56	0.96	1.92	4.81	0.32	0.32	1.28	0	5.13	0.96
Oppe river (Takasaka)	per individual(%)→		5.26	0	5.26	5.26	26.32	0	26.32	31.58	15.79	5.26	21.05	21.05	31.58	0
	per population(%)→		0.48	0	0.48	0.48	2.40	0	2.40	2.88	1.44	0.48	1.92	1.92	2.88	0

o	p	q	r	s	t	u	v	w	x	y	z	aa	bb	cc
34.62	42.31	46.15	3.85	0	3.85	3.85	76.92	19.23	30.77	50.00	15.38	7.69	23.08	7.69
2.88	3.53	3.85	0.32	0	0.32	0.32	6.41	1.60	2.56	4.17	1.28	0.64	1.92	0.64
47.37	15.79	36.84	21.05	0	0	0	89.47	5.26	26.32	52.63	15.79	0	21.05	0
4.33	1.44	3.37	1.92	0	0	0	8.17	0.48	2.40	4.81	1.44	0	1.92	0

dd	ee	ff	gg	hh	ii	jj	kk	ll	mm	nn	oo	pp	qq	rr
11.54	7.69	61.54	100	30.77	15.38	3.85	7.69	0	7.69	3.85	15.38	15.38	34.62	3.85
0.96	0.64	5.13	8.33	2.56	1.28	0.32	0.64	0	0.64	0.32	1.28	1.28	2.88	0.32
10.53	15.79	47.37	100	36.842	31.58	5.26	5.26	0	0	0	31.58	10.53	31.58	0
0.96	1.44	4.33	9.13	3.37	2.88	0.48	0.48	0	0	0	2.88	0.96	2.88	0

ss	tt	uu	vv	ww	xx	yy	zz	aaa
84.62	3.85	3.85	42.31	38.46	3.85	15.38	76.92	3.85
7.05	0.32	0.32	3.53	3.21	0.32	1.28	6.41	0.32
73.68	5.26	0	26.32	15.79	26.32	15.79	78.95	0
6.73	0.48	0	2.40	1.44	2.40	1.44	7.21	0

(M-2)

Table 2. Percentages of primer-1 bands in the *tenebrosusu* Daito-Bunnka-Univ., Kawajima and Oppe-river populations (M-1and M-2 express long-sized and short-sized markers, respectively; symbols of a to z, aa to zz and aaa are DNA bands).

Collection place ↓	Appearance ↓	Band→ (M−1)	a	b	c	d	e	f	g	h	i	j	k	l	m	n
Daito Bunka Univ.	per individual(%)→		0	0	14.86	9.46	75.68	6.76	0	1.35	39.19	5.41	0	1.35	78.38	6.76
	per population(%)→		0	0	1.30	0.83	6.60	0.59	0	0.12	3.42	0.47	0	0.12	6.84	0.59
Kawajima	per individual(%)→		0	0	33.33	66.67	33.33	0	0	66.67	0	0	0	0	100	0
	per population(%)→		0	0	2.50	5.00	2.50	0	0	5.00	0	0	0	0	7.50	0
Oppe river (Takasaka)	per individual(%)→		0	0	12.50	25.00	75.00	0	0	12.50	0	0	0	0	75.00	0
	per population(%)→		0	0	0.93	1.87	5.61	0	0	0.93	0	0	0	0	5.61	0

o	p	q	r	s	t	u	v	w	x	y	z	aa	bb	cc
78.38	82.43	0	0	100	0	1.35	4.05	36.49	6.76	1.35	9.46	45.95	77.03	1.35
6.84	7.19	0	0	8.73	0	0.12	0.35	3.18	0.59	0.12	0.83	4.01	6.72	0.12
66.67	100	0	0	100	0	0	0	66.67	33.33	0	0	0	100	0
5.00	7.50	0	0	7.50	0	0	0	5.00	2.50	0	0	0	8	0
87.50	100	0	0	100	0	25.00	0	12.50	62.50	0	12.50	0	100	12.50
6.54	7.48	0	0	7.48	0	1.87	0	0.93	4.67	0	0.93	0	7.48	0.93

dd	ee	ff	gg	hh	ii	jj	kk	ll	mm	nn	oo	pp	qq	rr
97.30	1.35	12.16	0	0	2.70	0	6.76	85.14	0	1.35	5.41	1.35	91.89	2.70
8.49	0.12	1.06	0	0	0.24	0	0.59	7.43	0	0.12	0.47	0.12	8.02	0.24
100	0.00	33.33	0	0	0	0	0	100	0	0	33.33	0.00	100	0
7.50	0.00	2.50	0	0	0	0	0	7.50	0	0	2.50	0.00	7.50	0
87.50	0.00	0	0	0	0	0	0	100	0	0	37.50	12.50	100	0
6.54	0.00	0	0	0	0	0	0	7.48	0	0	2.80	0.93	7.48	0

ss	tt	uu	vv	ww	xx	yy	zz	aaa
								(M−2)
2.70	55.41	2.70	12.16	36.49	2.70	41.89	0	0
0.24	4.83	0.24	1.06	3.18	0.24	3.66	0	0
0	0	0	33.33	66.67	33.33	66.67	0	0
0	0	0	2.50	5.00	2.50	5.00	0	0
0	62.50	12.50	50.00	75.00	75.00	0	0	12.50
0	4.67	0.93	3.74	5.61	5.61	0	0	0.93

Table 3. Differences between the *rufiventris* and the *tenebrosus* populations in the average frequencies of DNA bands (M-1and M-2 express long-sized and short-sized markers, respectively; symbols of a to z, aa to zz and aaa are DNA bands).

Band→	a	b	c	d	e	f	g	h	i	j	k	l	m	n
(M-1)														
Specific difference (%) →	0.56	0.32	1.02	2.32	2.42	0.28	2.16	1.83	0.26	0.24	1.60	0.92	2.64	0.28

o	p	q	r	s	t	u	v	w	x	y	z	aa	bb	cc
2.52	4.91	3.61	1.12	7.90	0.16	0.50	7.17	2.00	0.10	4.45	0.78	1.02	5.31	0.03

dd	ee	ff	gg	hh	ii	jj	kk	ll	mm	nn	oo	pp	qq	rr
6.55	1.00	3.54	8.73	2.96	2.00	0.40	0.36	7.47	0.32	0.12	0.16	0.77	4.78	0.08

ss	tt	uu	vv	ww	xx	yy	zz	aaa	
									(M-2)
6.81	2.77	0.23	0.53	2.27	1.42	1.52	6.81	0.16	

Table 4. Total and average differences in three local populations, Daito-Bunka-Univ.(Takasaka), Kawajima an Oppe-river (Takasaka), of *Bibio rufiventris* and *tenebrosus* and the two spscies (*Average is the total per species).

Species	Two populations	Difference(%)	
		Total	Average*
Bibio rufiventris	Kawajima and Oppe river(Takasaka)	38.78	19.39
Bibio tenebrosus	Kawajima and Oppe river(Takasaka)	46.59	23.29
	Daito Bunka Univ.(Takasaka) and Kawajima	52.26	26.13
	Daito Bunka Univ.(Takasaka) and Oppe river(Takasaka)	49.92	24.96
Bibio rufiventris and tenebrosus	Two species	120.24	60.12

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