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Source: Zoological Science, 19(10) : 1191-1196

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.1191

ZOOLOGICAL SCIENCE **19**: 1191–1196 (2002)

Mate Discrimination and Cuticular Hydrocarbons in *Drosophila elegans* and *D. gunungcola*

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ABSTRACT—In *Drosophila elegans*, two morphs are known, the brown-morph occurring from southern China to Indonesia and the black-morph occurring in the Ryukyu Islands, Japan, and Taiwan, and brown-morph populations at high altitudes in Indonesia at least sympatrically occur with a sibling species *D. gunungcola*. Sexual isolation has developed between the two morphs of *D. elegans* to some extent; females of the black-morph have a higher concentration of pentacosenes on cuticle than those of the brown-morph, and males of these morphs discriminate between the females based on this difference. In this study, it was examined whether sympatry and allopatry with *D. gunungcola* have resulted in the differentiation of mate recognition system in *D. elegans*. No significant difference was observed in the degree of mate discrimination between a sympatric pair of *D. elegans* and *D. gunungcola* and their allopatric pairs. Thus, no support was obtained for the above notion. Males of the brown- and black-morphs of *D. elegans* discriminate between females of own morphs and *D. gunungcola*. However, brown-morph males did not discriminate between females of the black-morph and *D. gunungcola*, and also black-morph males did not discriminate between females of the brown-morph and *D. gunungcola*. This may be attributed to that *D. gunungcola* females retained an intermediate level of pentacosenes between brown- and black-morph females.

Key words: character displacement, *Drosophila*, hydrocarbons, mate discrimination, mating propensity

INTRODUCTION

Evolution of ecological and reproductive characteristics is sometimes affected by the occurrence of sibling or closely related species. For example, a pair of species often differ more with each other where they are sympatric than where they are allopatric (Brown and Wilson, 1956). This phenomenon, called character displacement, is assumed to reflect the evolution to reduce interspecific competition (Grant, 1972, 1975) or prevent interspecific mating (Ehrman, 1965; Littlejohn, 1965; Littlejohn and Luftus-Hills, 1968; Dobzhansky *et al*., 1969; Wasserman and Koepfer, 1977; Waage, 1979; Coyne and Orr, 1989, 1997). In this paper, we examine whether or not the evolution of mate recognition system of *Drosophila elegans* Bock and Wheeler has been affected by the presence of its sibling *D. gunungcola* Sultana, Kimura and Toda.

There are two morphs in *D. elegans*, the black-morph which is distributed in the Ryukyu Islands, Japan, and Taiwan and the brown-morph which occurs in southern China, the Philippines and Indonesia (Lemeunier *et al*., 1984; Hirai and Kimura, 1997). We have reported that sexual isolation has developed between these two morphs to some extent; i.e., females of the black-morph (a strain from Iriomote-jima, Japan) have a higher concentration of 7- and 9-pentacosenes on cuticle than those of the brown-morph (a strain from Hong Kong, China) and males of these morphs discriminate between the females based on this difference (Ishii *et al*., 2001). The brown-morph of this species occurs sympatrically with a sibling species *D. gunungcola* at least at high altitudes in Indonesia (Sultana *et al*., 1999; Suwito *et al*., in press). *Drosophila elegans* and *D. gunungcola* mainly breed on *Ipomoea* flowers and their males hold mating territories on individual flowers (Hirai *et al*., 2000; Kimura and Hirai, 2001). Females of these two species sometimes co-occur in single flowers and interspecific courtship are often observed (Kimura, unpublished data). On the other hand, the black-morph of *D. elegans* is distributed in the regions where no other drosophilid species breeding on *Ipomoea*

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flowers is present (Hirai *et al*., 2000). Here, we examined mate discrimination and cuticular hydrocarbons of *D. gunungcola* and several strains of *D. elegans* to know whether or not the presence of *D. gunungcola* has affected the evolution of mate recognition system of the brown-morph of *D. elegans*.

MATERIALS AND METHODS

Flies

The experimental strains of *D. elegans* originated from a number of females collected in Sukarami, Indonesia (SK: 0°S in latitude), those in Hong Kong, China (HK: 22°N), those in Taipei, Taiwan (TP: 24°N), those in Iriomote-jima, Japan (IS: 24°N) and those in Okinawa, Japan (OH: 26°N). The SK and HK stains were brown-morph and the TP, IS and OH strains were black-morph. The strain of *D. gunungcola* originated from several females collected in Sukarami where the SK strain of *D. elegans* originated; the collecting localities were slightly different in altitude (1000 m for *D. elegans* and 1150 m for *D. gunungcola*). It is not known whether *D. gunungcola* is distributed in China or not because faunal survey is insufficient. However, *D. gunungcola* has not been observed in Hong Kong in our survey. In the localities where the black-morph strains of *D. elegans* were collected (<100 m in altitude), *D. gunungcola* has not been recorded. These strains were maintained in the laboratory for few years before experiments.

Experimental flies were reared on cornmeal-malt medium at 23°C under continuous light. Flies used in the experiments were sexed without anesthesia within 12 hr after eclosion and maintained for 7 days in glass vials (50 ml) containing food medium.

Cuticular hydrocarbons

About 200 virgin flies were immersed in *n*-hexane for 2 min to extract cuticular hydrocarbons. The hydrocarbons were purified by silicic acid column chromatography and each component was identified and quantified by gas-liquid chromatography and mass spectrometry (GC-MS) using JMS-AX500 (JEOL Ltd., Tokyo, Japan) (Katagiri *et al*., 1985). A capillary column (DB-1HT, 15m×0.25 mm) was run with He as carrier gas and programmed from 130 to 230°C at 5°C/min.

Mate discrimination

Mate discrimination was examined by courtship time in the choice-by-male tests. One male and two females of different strains or species were introduced into a glass vial (100 mm in height and 21 mm in diameter) containing food medium on the bottom with an aspirator without anesthesia. Duration of courtship exhibited to each females was measured until copulation had occurred or for 45 min if copulation did not occur. The index for mate discrimination in courtship (DIC) was calculated by the following formula;

$$DIC=(A-B)/(A+B)$$

where A and B are the number of replicates in which the males exhibit longer courtship to the females of strains A and B, respectively. DIC ranges from –1 to +1 and a value of zero indicates that males do not discriminate females. Females of the different morphs and species were discriminated by the coloration of body and legs. SK and HK females were discriminated by feeding either of them medium colored by brilliant blue prior to experiments. In this experiment, it was also recorded with which female the male mated.

RESULTS

Cuticular hydrocarbons

Table 1 shows the cuticular hydrocarbon compositions of the strains examined. In the female, the concentration of pentacosenes was 2.5 to 7 times higher in the IS, TP and OH strains (the black- morph: about 13%) than in the HK and SK strains (the brown-morph: about 2–5%). In the brown-morph, the concentration of pentacosenes was about 2 times higher in SK females than in HK females. Females of *D. gunungcola* retained a similar concentration (about 6%) of pentacosenes with those of the SK strain, but the

Table 1. Cuticular hydrocarbon compositions (%) in the experimental strains of *D. elegans* (HK, SK, IS, TP OH) and *D. gunungcola* (GC).

	alkane						alkene				
	C_{21}	C_{23}	C_{25}	C_{27}	C_{29}	Total	$C_{23:1}$	$C_{25:1}$	$C_{27:1}$	$C_{29:1}$	Total
Female											
HK	17.8	14.7	1.7	3.1	2.5	39.8	56.0	2.7	0.7	0.8	60.2
SK	8.8	19.1	2.0	2.4	2.9	35.1	57.2	5.3	1.6	0.8	64.9
IS	12.3	14.3	2.1	2.8	2.6	34.1	48.3	14.4	1.7	1.5	65.9
TP	10.3	17.0	2.2	2.8	2.2	34.6	50.2	12.4	1.4	1.4	65.4
OH	10.1	17.1	1.9	2.6	2.7	34.4	52.0	11.1	0.9	1.7	65.6
GC	7.2	14.2	1.4	3.1	1.9	27.7	61.0	6.1	2.9	2.3	72.3
Male											
HK	9.4	14.1	3.8	4.4	1.6	33.3	56.4	2.5	5.7	2.1	66.7
SK	5.8	15.2	3.4	3.6	0.7	28.7	56.3	6.0	6.7	2.2	71.3
IS	5.9	13.9	3.3	3.9	1.3	28.3	55.6	5.3	8.6	2.3	71.7
TP	5.5	13.3	3.7	3.7	3.9	30.0	55.0	5.7	7.3	2.0	70.0
OH	5.9	14.3	3.1	3.8	1.4	28.4	57.8	5.5	6.5	1.8	71.6
GC	4.8	11.7	2.1	4.0	1.1	23.6	58.1	5.2	9.7	3.3	76.4

C_{21} (heneicosane), C_{23} (tricosane), C_{25} (pentacosane), C_{27} (heptacosane), C_{29} (nonacosane), $C_{23:1}$ (tricosenes), $C_{25:1}$ (pentacosenes), $C_{27:1}$ (heptacosenes), $C_{29:1}$ (nonacosenes).

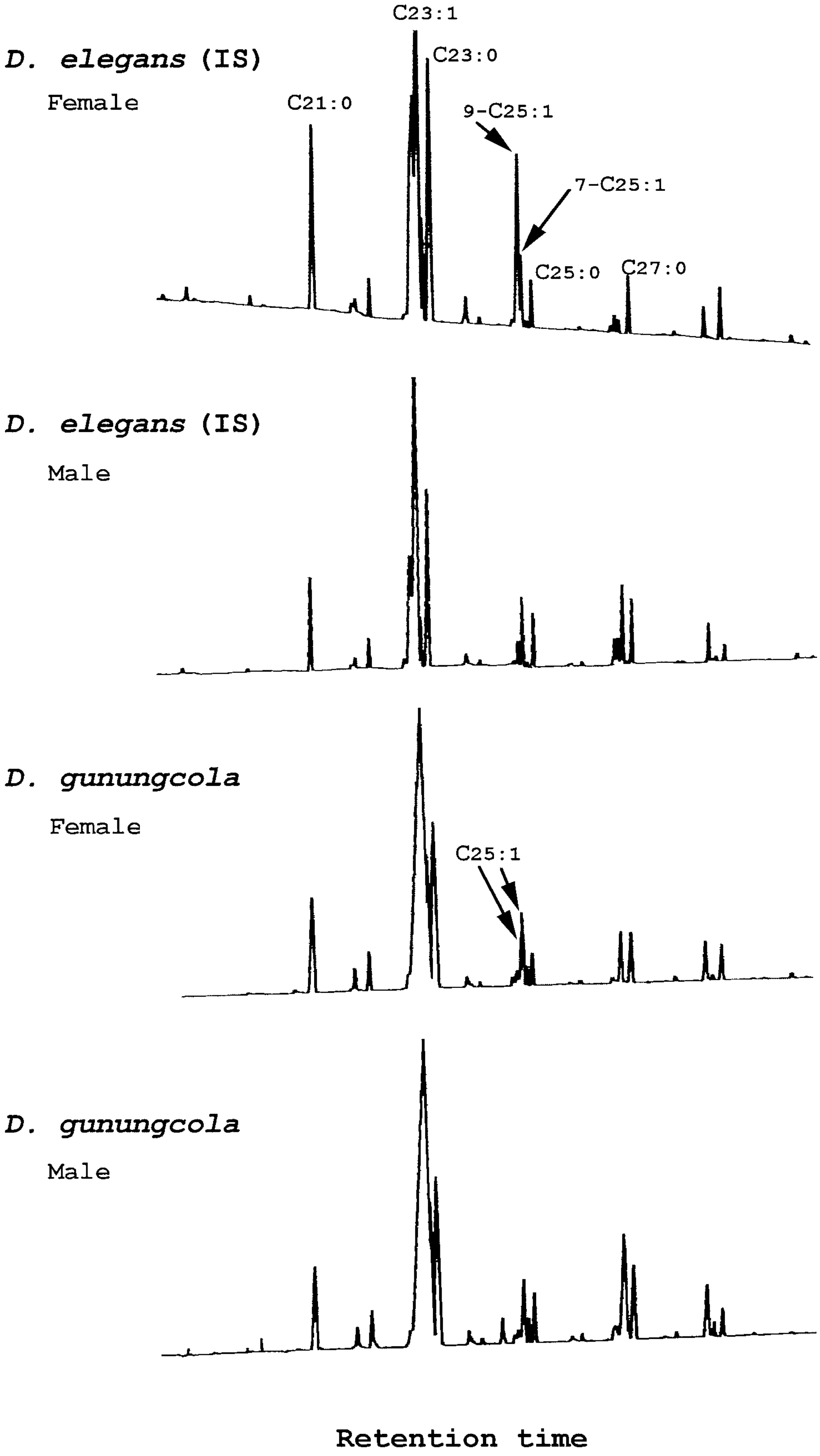

Fig. 1. Gas chromatograms of female and male *Drosophila elegans* (the IS strain) and *D. gunungcola*. $C_{21:0}$ (heneicosane), $C_{23:1}$ (tricosenes), $C_{23:0}$ (tricosane), 9-$C_{25:1}$ (9-pentacosene), 7-$C_{25:1}$ (7-pentacosene), $C_{25:0}$ (pentacosane) and $C_{27:0}$ (heptacosane).

molecular composition of pentacosenes differed between these two species; 9-pentacosene was dominant in *D. elegans* (Fig. 1 and also see Ishii *et al.*, 2001), while a different molecular species was major in *D. gunungcola* (Fig. 1). The concentration of heptacosenes and nonacosenes was lower in females of the HK strain than in those of other strains or species. Concomitantly with the difference in the concentration of these alkenes, the tricosene concentration differed among these strains and species. However, the difference in tricosene concentration was only 15–20% because tricosenes are dominant.

In *D. elegans* males, the concentration of pentacosenes was somewhat lower and that of heneicosane was somewhat higher in the HK strain than in the SK, IS, TP or OH strains. The concentration of heptacosenes and nonacosenes was somewhat higher in *D. gunungcola* males than in *D. elegans* males.

Mate discrimination between *D. elegans* strains

HK (brown-morph) males exhibited longer courtship to HK females more frequently than to TP or IS (black-morph) females (Table 2: #1 and #2), and males of the IS and TP strains exhibited longer courtship to IS or TP females more frequently than to HK females (Table 2: #7 and #10). In these experiments, the percentage of males which exhibited courtship only to females of their own strains was high (44–60%), and the percentage of males which exhibited courtship only to females of the different morph was low (13–24%). SK males (brown-morph) also exhibited longer courtship to SK females more frequently than to IS females (Table 2: #5). In this experiment, however, the percentage of males which exhibited courtship only to SK females was not high (23%). On the other hand, HK, SK and IS males did not discriminate between HK and SK females (Table 2: #3, #6 and #9), and HK and IS males did not discriminate

Table 2. Results of choice-by-male test with the black (IS, TP) and brown (HK, SK) morph strains of *D. elegans.*

	Male	Female	Males that exhibited longer courtship to A female			Males that exhibited longer courtship to B female			
		A, B	N	No. mated	No. mated with A female	N	No. mated	No. mated with B female	DIC
#1	HK	HK, IS	25 (18)	23	21	5 (4)	4	3	$0.67^{**, \dagger\dagger}$
#2	HK	HK, TP	21 (15)	20	17	9 (4)	9	7	$0.40^{\dagger}$
#3	HK	HK, SK	20 (11)	20	19	11 (0)	9	3	0.29
#4	HK	SK, IS	14 (5)	13	7	16 (8)	16	10	–0.07
#5	SK	SK, IS	23 (7)	16	6	7 (5)	6	6	$0.53^{*, \dagger\dagger}$
#6	SK	SK, HK	15 (6)	14	7	15 (8)	14	13	0.00
#7	IS	IS, HK	37 (30)	33	31	17 (12)	13	11	$0.35^{\dagger}$
#8	IS	IS, SK	17 (13)	14	13	13 (0)	10	1	0.13
#9	IS	HK, SK	16 (11)	14	14	14 (7)	13	7	0.07
#10	TP	TP, HK	40 (27)	40	35	23 (11)	20	14	$0.27^{\dagger}$

Number in parenthesis indicates the number of males that exhibited courtship only to the corresponding female.
Significantly deviated from random courtship (χ^2-test with sequential Bonferroni correction, $^{*}P<0.05$, $^{**}P<0.01$, and χ^2-test without correction, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$).

Table 3. Results of choice-by-male test with *D. elegans* (IS, HK and SK) and *D. gunungcola* (GC).

	Male	Female	No. of males exhibited longer courtship to		DIC	No. of males mated with	
		A, B	A females	B felames		A females	B females
#1	HK	HK, GC	29	14	$0.35^{\dagger}$	39	0
#2	HK	IS, GC	14	16	–0.07	16	0
#3	SK	SK, GC	25	5	$0.67^{**, \dagger\dagger}$	21	0
#4	IS	IS, GC	28	12	$0.40^{*, \dagger\dagger}$	24	0
#5	IS	HK, GC	17	13	0.13	22	0
#6	GC	GC, HK	28	11	$0.44^{*, \dagger\dagger}$	36	0
#7	GC	GC, SK	25	5	$0.67^{**, \dagger\dagger}$	26	0
#8	GC	GC, IS	27	3	$0.80^{**, \dagger\dagger}$	23	0

Significantly deviated from random courtship (χ^2-test with sequential Bonferroni correction , $^{*}P<0.05$, $^{**}P<0.01$, and χ^2-test without correction, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$).

between SK and IS females (Table 2: #4 and #8).

HK, IS and TP females copulated at high rates (>60%) if they received longer courtship, but SK females copulated less frequently (<50%) even if they received longer courtship. In Experiment #5, for example, copulation occurred with SK females in 6 case but with another (IS) females in 10 cases among 23 cases in which SK females received longer courtship (copulation did not occur in 7 cases).

Mate discrimination between *D. elegans* and *D. gunungcola*

Copulation did not occur at all between *D. elegans* and *D. gunungcola*, although males of both species often exhibited courtship to heterospecific females (Table 3). Males of the HK, SK and IS strains of *D. elegans* usually showed longer courtship to females of their own strains than to *D. gunungcola* females (Table 3: #1, #3 and #4), and males of *D. gunungcola* showed longer courtship more frequently to conspecific females than to females of the HK, SK and IS strains of *D. elegans* (Table 3: #6, #7 and #8). No significant difference was observed in DIC between a sympatric pair (*D. gunungcola* – the SK strain of *D. elegans*) and allopatric pairs (*D. gunungcola* – the HK or IS strain) (χ^2 test with sequential Bonferroni correction, $P>0.05$). On the other hand, males of the HK strain did not discriminate between females of the IS strain and *D. gunungcola* (Table 3: #2), and also males of the IS strain did not discriminate between females of the HK strain and *D. gunungcola* (Table 3: #5).

DISCUSSION

Mating propensity in *D. elegans*

In the present choice-by-male tests, HK (brown-morph), IS (black-morph) and TP (black morph) females copulated with high frequencies (>60%) if they received longer courtship, but SK (brown-morph) females copulated with relatively low frequencies (<50%) even if they received longer courtship. This was true in the case where SK females received longer courtship from males of the own strain (Table 2: # 5 and #6). It is therefore concluded that the mating propensity was lower in SK females than in HK, IS or TP females.

The female mating propensity seems to affect male mating behaviors. For example, in Experiments #3 and #8 (Table 2), a number of HK or IS males exhibited longer courtship to SK females, but none of them exhibited courtship only to SK females. This would be due to that males changed the object of courtship from SK females to another females if they first exhibited courtship to SK females, since SK females were reluctant to copulate (i.e., their mating propensity was low). Thus, the results of the choice tests using SK females may be somewhat biased.

Mate discrimination between *D. elegans* strains

Our previous study revealed that premating isolation between the HK and IS strains bases on the difference in female cuticular hydrocarbon composition (Ishii *et al*., 2001); i.e., females of the IS strain have a higher concentration of pentacosenes than those of the HK strain, and males of these strains discriminate the mates based on this difference. In this study, it appeared that the percentage of pentacosenes was also high in females of the other black-morph strains (TP and OH) and low in females of the other brown-morph strain (SK). In addition, about a half (44–60%) of males exhibited courtship only to females of their own strains and a majority (63–83%) of them exhibited longer courtship to females of their own strains more frequently than to females of different morphs in the choice tests with HK, IS and TP strains, indicating that males prefer females of their own strains rather than females of different morphs. In the choice tests between the SK and IS strains, SK males also exhibited longer courtship more frequently to SK females than to IS females. On the other hand, HK and IS males did not discriminate between SK and IS females. However, the results of these choice tests using SK females may be biased due to the low mating propensity of SK females, as noted previously.

Sexual isolation between *D. elegans* and *D. gunungcola*

If sibling species with similar sexual signals encounter in nature, mate recognition system (sexual signals and/or ability to discriminate signals) may change in either or both species to avoid interspecific mating (Brown and Wilson, 1956). In fact, it has been observed that calling sounds of *Hyla* (Littlejohn and Luftus-Hills, 1968) and wing coloration of *Calopteryx* (Waage, 1979) differ more where they occur sympatrically than where they occur allopatrically. The present study species, *D. elegans* and *D. gunungcola* are reported to occur sympatrically at least at high altitudes in Indonesia (Sultana *et al*., 1999; Suwito *et al*., in press). Therefore, there is a possibility that the presence with *D. gunungcola* has affected the evolution of mate recognition system of the sympatric populations of *D. elegans*. If this is the case, mate discrimination would be stronger between *D. gunungcola* and a strain of *D. elegans* from a sympatric population (SK) than between *D. gunungcola* and strains of *D. elegans* from allopatric populations (HK or IS). In the present experiment, however, there observed no significant difference in the degree of mate discrimination between sympatric and allopatric pairs. In addition, *D. gunungcola* did not mate not only with the SK strain but also with the HK or IS strains. Thus, no support was obtained for the notion that the presence of *D. gunungcola* affects the mate discrimination system of *D. elegans*. This also indicates that the differentiation of mate recognition system between the brown- and black-morphs of *D. elegans* occurred independent of the presence of *D. gunungcola*.

Copulation did not occur between *D. elegans* and *D. gunungcola*, although males of both species sometimes exhibited courtship to heterospecific females. This may suggest that females of these species discriminate between conspecific and heterospecific males. If this is the case, their discrimination would be based on signals other than cuticular hydrocarbons, because male cuticular hydrocarbon profile was not so much different between these two species. However, no clear difference has not been observed in male courtship behaviors between these species.

Males of the HK and IS strain of *D*. *elegans* discriminate between females of own strains and those of *D*. *gunungcola*. However, HK males did not discriminate between females of the IS strain and those of *D*. *gunungcola*, and also IS males did not discriminate between females of the HK stain and those of *D*. *gunungcola*. This may be attributed to that *D*. *gunungcola* females retained an intermediate level of pentacosenes between HK and IS females, although molecular species of major pentacosenes differ between these two species.

ACKNOWLEDGEMENTS

This work was partly supported by Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (No. 10304062 and No. 11691161) and by Japan Society for the Promotion of Science (Core University Program entitled “Environmental management of tropical wetland ecosystem in Southeast Asia”).

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(Received May 24, 2002 / Accepted August 7, 2002)