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Identifications of Some Species of the Genus *Rhopalomyia* (Diptera: Cecidomyiidae) Inducing Galls on *Artemisia* (Asteraceae) in South Korea*

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Abstract. In 2003, four species of the genus *Rhopalomyia* (Diptera: Cecidomyiidae) were collected from *Artemisia* species (Asteraceae) in South Korea. To identify these species, morphological features, gall appearance, host plant species, and DNA sequence data of each species were compared with those of respective *Rhopalomyia* species that induce similar galls on *Artemisia* species in Japan. Three of the four South Korean *Rhopalomyia* species were identical with Japanese *Rhopalomyia* species, respectively; they are *R. struma*, *R. yomogicola*, and *Rhopalomyia* sp. that induces globular axillary bud galls. In the case of *Rhopalomyia* sp., DNA sequence data indicated that speciation could be initiated by geographical isolation prior to host shifting. Remaining one species that was collected from terminal bud galls on *Artemisia capillaris* in South Korea was different from the Japanese species that induces a similar gall on *A. princeps* and *A. montana*. This South Korean gall midge will be described as a new species elsewhere in the future.

Key words: *Rhopalomyia*, Cecidomyiidae, *Artemisia*, gall, Korea, speciation.

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Introduction

About 250 species of the genus *Artemisia* Linnaeus (Asteraceae) have been recorded in the world. In Japan, there are 31 *Artemisia* species, consisting of 24 in the section *Artemisia* and seven in the section *Dracunculus* (Satake *et al.*, 1981; Hotta *et al.*, 1989). Twelve Japanese gall midge species of the genus *Rhopalomyia* Rübsaamen, 1892 (Diptera: Cecidomyiidae) are known to utilize *Artemisia feddei* Léveillé et Vaniot, *Artemisia montana* (Nakai) Pampan, and *Artemisia princeps* Pampan in the section *Artemisia*, and *Artemisia capillaris* Thunberg and *Artemisia japonica* Thunberg in the section *Dracunculus* as their host plants (Yukawa & Masuda, 1996).

From the Korean Peninsula and surrounding islands (Korea, hereafter), at least 30 gall-inducing species of Cecidomyiidae (Diptera) have been recorded (Saitô, 1932; ESK & KSAE, 1994; Yukawa & Masuda, 1996; Kodoi *et al.*, 2003). Four species of them have been identified as members of *Rhopalomyia* based on similarities of the appearance and structure of their galls to those induced by Japanese gall midges on *Artemisia* species. They are *Rhopalomyia struma* Monzen, 1937, *Rhopalomyia giraldii* Kieffer et Trotter, 1900, *Rhopalomyia yomogicola* (Matsumura, 1931), and *Rhopalomyia artemisiae* (Bouché, 1834).

In many cases, the appearance and structure of midge galls are specific to gall-midge species, and each gall is induced on particular plant taxa. Therefore, the galls are regarded as an extended phenotype of respective gall-midge species, except for some polyphagous species (e.g., Yukawa *et al.*, 2003; Uechi *et al.*, 2003; Uechi *et al.*, 2004). Thus, the identification of these Korean *Rhopalomyia* species based on host plant and gall information seems to be reliable. However, it would be better to confirm the identification at the DNA level because there are some instances in which gall midges are different even when they induce the same sort of gall on the same host organ and species (Uechi *et al.*, unpublished data).

In addition, comparison between the variation of haplotypes from different localities and that of haplotypes from different host plants will provide us with useful information about which of the factors, geographical isolation or host plant shift, primarily affect speciation of gall midges.

This paper intends to identify four species of *Rhopalomyia* that were collected from Sunchon, Jeonnam and its vicinity, South Korea. The identification was based not only on the morphological features of gall midges and the appearance of their galls but also on DNA sequence data, with which influence of the two factors, geographical isolation and host plant shift, on speciation were compared.

Materials and Methods

Collection and preservation of specimens

Three sorts of gall on *A. princeps*, and one on *A. capillaris* were collected on May 28, 2003 from Sunchon, Jeonnam and its vicinity, the most southern parts of the Korean Peninsula. Among them, two sorts on *A. princeps* have been recorded from Korea; one is similar to a stem gall (Photograph Number: D-102 in Yukawa & Masuda, 1996) induced by *R. struma* in Japan and another is to a subconical leaf gall (D-112) induced by *R. yomogicola* in Japan. Remaining one on *A. princeps* is similar to an axillary bud gall (D-110) induced by *Rhopalomyia* species in Japan, but it was not recorded previously from Korea. The last one on *A. capillaris* was not found previously both in Japan and Korea, although its appearance is similar to a terminal bud gall induced by *Rhopalomyia abdominalis* Shinji, 1938 on *A. montana* or to that (D-108) by *Rhopalomyia iwatensis* Shinji, 1938 on *A. japonica* and *A. princeps* in Japan.

Some of these galls were dissected under a binocular microscope and unparasitized mature midge larvae and pupae were picked out of the galls and kept in 70-75 % ethanol for morphological studies or in 99.5 % acetone for DNA analysis. Remaining galls were maintained in plastic containers (10 cm in diameter, 6 cm in depth) to rear adult midges. Emerged adults were also put into 70-75 % ethanol or 99.5 % acetone.

Morphological study and identification of Rhopalomyia *species*

At the time of dissecting galls, appearance and inner structure of the galls collected from South Korea were examined. The South Korean gall midges obtained from these galls were compared, respectively, with Japanese *Rhopalomyia* species inducing galls that are similar to those collected from South Korea.

The full-grown larvae, pupae, and adults that had been stored in 70-75 % ethanol were mounted on slides in Canada balsam for microscopic study, based on the techniques outlined in Gagné (1989). These specimens were examined with a bright-field and phase-contrast microscopy, a Nikon ECLIPSE E400 microscope. Special attention was paid to dorsal and ventral papillae on larval prothorax, larval terminal segment, and pupal antennal sheath and frontal area, since they frequently exhibit species-specific features.

The slide-mounted specimens examined in this study are kept in the collection of Entomological Laboratory, Faculty of Agriculture, Kyushu University, Fukuoka, Japan.

DNA extraction, amplification, and sequencing

At least three individuals of each species were used for DNA analysis. For every individual, total DNA was extracted from the whole body with the Dneasy tissue kit

(Qiagen, Japan), following the manufacturer's instructions. A region of the cytochrome oxidase subunit I (COI) gene of mtDNA was amplified by PCR following the methods described by Yukawa *et al.* (2003). DNA of each specimen was amplified using the following primers: forward; 5'-GGATCACCTGATATAGCATTCCC-3' (COIS) and reverse; 5'-CCCGGTAAAATTAAAATATAAACTTC-3' (COIA). These primers have been effectively used for many gall midges to determine intra- and inter-specific relations (e.g., Shirota *et al.*, 1999; Yukawa *et al.*, 2003; Uechi *et al.*, 2003; Uechi *et al.*, 2004). The amplified COI gene fragment of mtDNA was 439 bp long. This region corresponded to the bases 1752-2190 of the genome of *Drosophila yakuba* Burla (Diptera: Drosophilidae) (Clary & Wolstenholme, 1985).

The amplified products were purified with the QiAquick PCR purification kits (Qiagen, Japan) following the manufacturer's instructions. The purified products were sequenced by the dideoxy-nucleotide cycle sequencing procedure with the Dye-Terminator cycle sequencing kit (Perkin-Elmer, Warrington, UK) and TGRADIENT thermal cycler (Biometrica). Sequencing electrophoresis was done on an 11 % Long Ranger™ gel with a LIC-4200S-2 automated DNA sequencer (Aloka Co. Ltd., USA). Both strands of the PCR products were sequenced.

Molecular phylogenetic analysis

Phylogenetic analysis was conducted by the neighbor-joining (NJ) method (Saitou & Nei, 1987) using the software package PHYLIP ver. 3.573c (Felsenstein, 1993). The resulting trees were evaluated by the bootstrap test (Efron, 1982; Felsenstein, 1985) based on 1,000 replications.

As outgroup taxa in the above analysis, the following two gall-midge species (Diptera: Cecidomyiidae) were used (Table 1): *Contarinia okadai* (Miyoshi) infesting the blossoms of *Citrus* spp. (Rutaceae) and *Asphondylia yushimai* Yukawa et Uechi, an important pest of soybean in Japan.

Nucleotide sequence data obtained in this study are registered with DDBJ (DNA Data Bank of Japan), EMBL (European Molecular Biology Laboratory), and GenBank nucleotide sequence databases (Table 1).

Results

Rhopalomyia struma*-like gall midge inducing stem galls in South Korea*

Eight haplotypes were found in the sequential variations of *R. struma* in Japan and one haplotype in the South Korean *R. struma*-like gall midge (Fig. 1). Among the eight haplotypes of *R. struma*, there were one (0.23 %) to 12 (2.73 %) bp differences, and at most three differences in the 146 deduced amino acid residues. The South Korean

haplotype was one (0.23 %) to eight (1.82 %) bp different from the haplotypes of *R. struma* and there were one to three differences in the amino acid residues. However, the South Korean haplotype was included in the clade of *R. struma*. The monophyly of the clade including the South Korean haplotype and Japanese *R. struma* was supported by a

Table 1. Specimens used for DNA analysis: two species of *Rhopalomyia* galling on *Artemisia* in South Korea and Japan and two outgroup species of gall midges, *Contarinia okadai* and *Asphondylia yushimai*.

Gall midge	Host plant	Collection site	Isolation name	Accession no.*
Rhopalomyia sp.	*A. princeps*	Rifu, Miyagi, Japan	mmtbprimyg01	AB162385
		Hakozaki, Fukuoka, Japan	mmtbprifuk02	AB162386
			mmtbprifuk03	AB162387
		Yufuin, Oita, Japan	mmtbprioit04	AB162388
		Kokonoe, Oita, Japan	mmtbprioit05	AB162389
			mmtbprioit06	AB162390
		Ume, Oita, Japan	mmtbprioit07	AB162391
		Kitagawa, Miyazaki, Japan	mmtbprimyz08	AB162392
			mmtbprimyz09	AB162393
			mmtbprimyz10	AB162394
		Sunchon, Jeonnam, South Korea	mmtbprikor11	AB162395
			mmtbprikor12	AB162396
			mmtbprikor13	AB162397
R. struma	*A. montana*	Bibai, Hokkaido, Japan	kukimonhok14	AB162398
			kukimonhok15	AB162399
	A. princeps	Higashinaruse, Akita, Japan	kukipriaki16	AB162400
			kukipriaki17	AB162401
		Kureha, Toyama, Japan	kukipritym18	AB162402
			kukipritym19	AB162403
		Bunkyo, Fukui, Japan	kukiprifki20	AB162404
		Misumi, Shimane, Japan	kukiprismn21	AB162405
			kukiprismn22	AB162406
			kukiprismn23	AB162407
		Kitakyushu, Fukuoka, Japan	kukiprifuk24	AB162408
			kukiprifuk25	AB162409
			kukiprifuk26	AB162410
		Yufuin, Oita, Japan	kukiprioit27	AB162411
			kukiprioit28	AB162412
			kukiprioit29	AB162413
			kukiprioit30	AB162414
		Kitagawa, Miyazaki, Japan	kukiprimyz31	AB162415
			kukiprimyz32	AB162416
			kukiprimyz33	AB162417
		Sunchon, Jeonnam, South Korea	kukiprikor34	AB162418
			kukiprikor35	AB162419
			kukiprikor36	AB162420
Out group				
Contarinia okadai	*Citrus iyo*	Tachibana, Yamaguchi, Japan	ConokaYG-T267	AB105485
Asphondylia yushimai	*Glycine max*	Chikushino, Fukuoka, Japan	SoyFuk15	AB085787

* Nucleotide sequence data used in this study are available from DDBJ, EMBL, and GenBank.

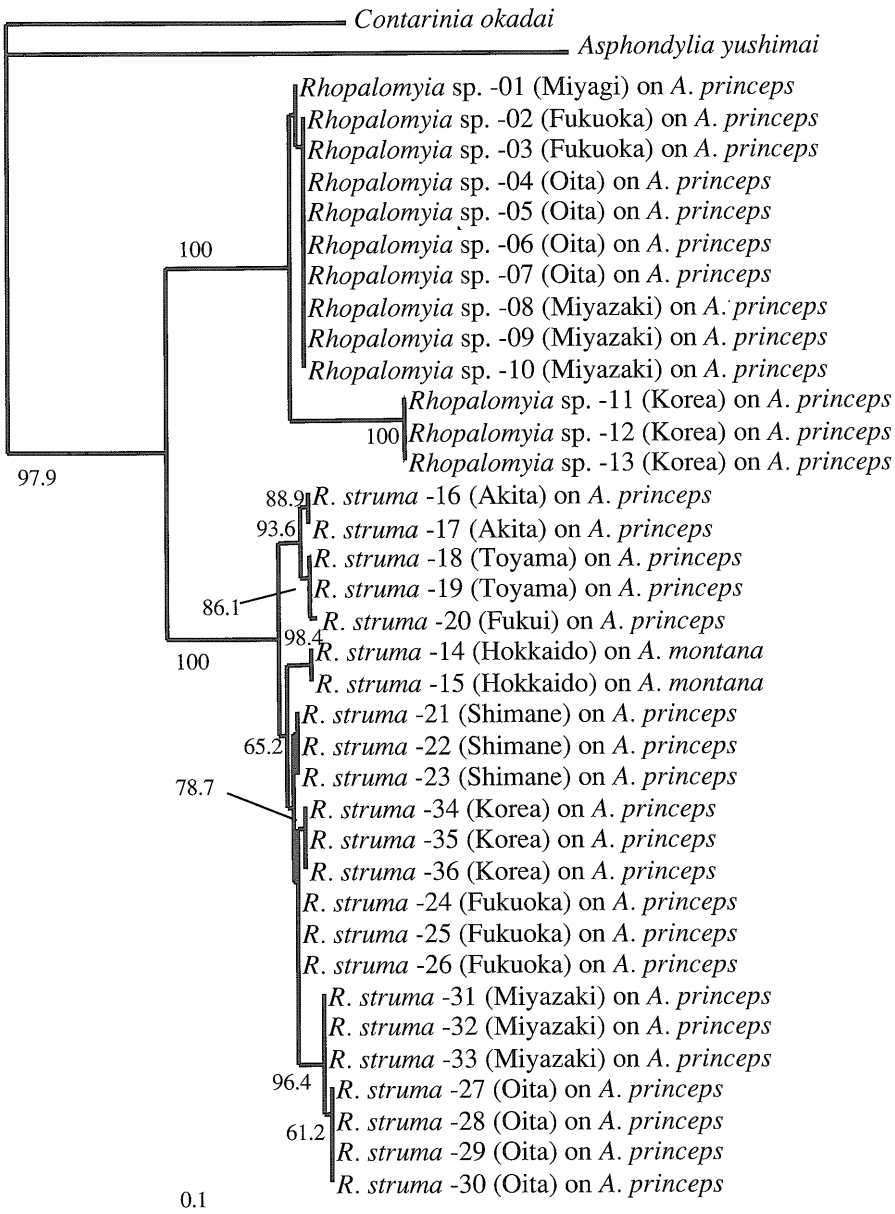
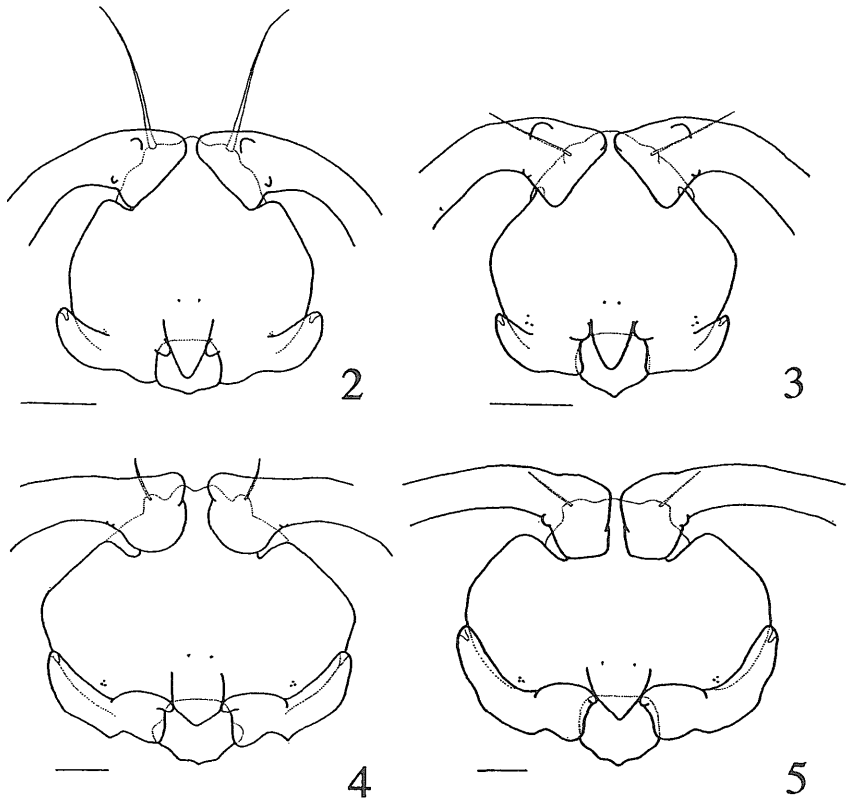


Fig. 1. NJ-tree based on 439 bp of the mtDNA COI gene for two *Artemisia* gall midges, *Rhopalomyia struma* and *Rhopalomyia* sp., from South Korea and Japan. Bootstrap values are indicated for nodes gaining more than 60 % support (1,000 replications). *Contarinia okadai* and *Asphondylia yushimai* were used as outgroup species. Sample numbers correspond to the respective isolation names registered in DNA database (see Table 1).



Figs. 2-5. Ventral view of pupal head (Scale bar = 0.1 mm). 2: *Rhopalomyia struma* from South Korea, 3: *Rhopalomyia struma* from Japan, 4: *Rhopalomyia* sp. inducing axillary bud galls in South Korea, and 5: *Rhopalomyia* sp. inducing axillary bud galls in Japan.

100 % bootstrap value (Fig. 1). Morphological features, particularly those of pupal frontal area and the basal portion of antenna in pupa, also supported the identity between the South Korean (Fig. 2) and Japanese species (Fig. 3).

Rhopalomyia yomogicola-*like gall midge inducing subconical leaf galls in South Korea*

At least 23 haplotypes were found in the sequential variations of *R. yomogicola* in Japan and three in the South Korean *R. yomogicola*-like gall midge. These South Korean haplotypes were included here and there in small, scattered clades consisting of a few Japanese haplotypes. NJ-tree was not indicated in this paper for *R. yomogicola* because further DNA analysis in different regions is required to confirm if these

scattered clades are unified into a main clade of *R. yomogicola*.

Rhopalomyia *sp. inducing globular axillary bud galls in South Korea*

As to the Japanese *Rhopalomyia* species that induces globular galls on the axillary buds, there were two haplotypes in the individuals collected from Miyagi Prefecture and various prefectures in Kyushu (Fig. 1). Only one haplotype was found in the individuals of the South Korean *Rhopalomyia* species that induces globular axillary bud galls. Between the two haplotypes of the Japanese *Rhopalomyia* species, there were one (0.23 %) bp difference, and only one difference in the 146 deduced amino acid residues. The South Korean haplotype was 14 (3.19 %) to 15 (3.42 %) bp different from the Japanese haplotypes and there were seven to eight differences in the amino acid residues. However, the South Korean haplotype was included in the clade of the Japanese *Rhopalomyia* species. The monophyly of the clade including the South Korean haplotype and the Japanese *Rhopalomyia* species was supported by a 100 % bootstrap value (Fig. 1).

There were slight morphological differences in pupa between the South Korean and the Japanese species: base of antenna slightly developed anteriorly into a rounded lobe in the South Korean species (Fig. 4), while undeveloped and gently rounded in the Japanese species (Fig. 5).

Rhopalomyia *sp. inducing terminal bud galls in South Korea*

The *Rhopalomyia* species that induces terminal bud galls on *A. capillaris* in South Korea was morphologically different in the basal portion of pupal antennal horn from *R. abdominalis* on *A. montana* and *R. iwatensis* on *A. japonica* and *A. princeps* in Japan. Sequencing data also supported the difference. These data are not indicated in this paper, because the South Korean species will be described as a new species in a separate paper.

Discussion

Morphological features and DNA analysis indicated that three of the four South Korean *Rhopalomyia* species were identical with the Japanese *Rhopalomyia* species on *Artemisia*, respectively. They were *R. struma*, *R. yomogicola,* and the *Rhopalomyia* species that induces globular axillary bud galls. These results revealed that the species identification based on host plant and gall information was reliable at least for the interrelation between *Artemisia* and *Rhopalomyia* in South Korea and Japan.

Differences in the sequential variations of *R. struma* were very small between South Korean and Japanese populations and between different host plants, *A. princeps* and *A. montana* (Fig. 1). This means that diversification does not proceed in *R. struma* between

the two areas and between the different host plants.

In contrast, there were relatively big differences between South Korean and Japanese populations in the sequential variations of the *Rhopalomyia* species that induces globular axillary bud galls on *A. princeps* (Fig. 1). In addition, there were slight morphological differences in the base of pupal antenna between the South Korean and the Japanese populations (Figs. 4, 5). Such differences do not mean that they are different species at the moment, but may suggest that they began to speciate.

In Japan, the *Rhopalomyia* species is known to induce the same sort of gall also on *A. montana* (Yukawa & Masuda, 1996), and preliminary DNA analysis showed relatively small sequential differences between populations on *A. princeps*, *A. montana*, and *A. feddei* (Ganaha *et al.*, unpublished data). In the case of the *Rhopalomyia* species, diversification could be initiated by geographical isolation prior to host plant shift.

As a result, the number of *Rhopalomyia* species in Korea stood at six, although the two previously recorded species, *R. giraldii* and *R. artemisiae*, could not be examined in this study.

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