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Sexual dimorphism documented in *Reptalus iguchii* (Matsumura) (Hemiptera: Fulgoromorpha: Cixiidae) with a description of males

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Abstract

Reptalus iguchii (Matsumura 1914) comb. nov., previously known from female specimens, is recognized for the first time in the Republic of Korea. This species is sexually dimorphic, based on observations of a pair *in copula*. Detailed morphological characters of both male and female were studied and are illustrated. Also, sequence data from ribosomal internal transcribed spacer 2 (ITS2) and cytochrome oxidase subunit I (COI) genes were used to distinguish males and females of the only other congener known from Korea, *R. quadricinctus* (Matsumura 1914). *Artemisia* and *Prunus* are recorded as potential hosts in the Republic of Korea for the first time.

Key words: CO1, fulgoroidea, ITS2, *Oliarus iguchii*, *Reptalus*, taxonomy.

Introduction

The genus *Reptalus* Emeljanov (Cixiidae: Pentastirini) includes 29 recognized species distributed in Europe, Asia (except southern Asia) and northern Africa (Bourgoin 2011). This genus is subdivided into two subgenera, *Reptalus* s. str. and *Trepalus* Emeljanov, based on the distribution of subapical setae (platellae) on the hind tarsi and characters of genital structures (Emeljanov 1995). To date, one species in this genus is known from Korea, *R. quadricinctus* (Matsumura 1914) (Kwon & Huh 2001). We identified a second species, *R. iguchii* (Matsumura 1914) for the first time from the Republic of Korea.

Field observations suggested sexual dimorphism for *R. iguchii*. Identification at the species level for the genus is based on morphology of the genitalia, but no morphological description for *R. iguchii* males has been available. Therefore, we were uncertain whether we were dealing with males and females of the same species or with a closely related species. Molecular markers can be helpful in such circumstances. Two commonly used markers, cytochrome oxidase subunit I (COI) and internal transcribed spacer 2 (ITS2) were chosen for species discrimination. Furthermore, these markers have worked well for other species within *Reptalus* (Bertin *et al*. 2010). We applied these molecular markers and compared results with those obtained from *R. quadricinctus* the only other representative of the genus in Korea and thus the only species that could probably be mistaken for *R. iguchii*.

Materials and methods

Sample collection

Adults of *R. iguchii* (n = 48 males, n = 33 females) were collected by sweeping vegetation in various regions of the Republic of Korea between 1979 and 2010. During the last year, field surveys were mainly targeted at plants known as potential hosts of *Reptalus* species (Holzinger *et al*. 2003; Mazzoni 2005; Bertin *et al*. 2010). Adults of *R. iguchii* were collected from *Prunus* (n = 9 males, n = 8 females) and *Artemisia* (n = 14 males, n = 12 females) plants. Adults of *R. quadricinctus* (n = 18 males, n = 22 females) were collected from a potato field by sweeping. Both species were collected at different areas of Gyeongsangbuk-do province in the Republic of Korea. Adult specimens of both sexes of

R. iguchii and *R. quadricinctus* were preserved separately in absolute ethanol and kept at −20°C until their morphological identification and/or DNA extraction.

Morphological study

Specimens from old (1979–1998) and recent (2010) collections were individually identified under a stereo microscope and morphological features were used to determine family, genus and subgenus. For species identification, both male and female genitalia were dissected and studied. All measurements are in millimeters. Photographs were imported into Adobe Photoshop CS3 for labeling and plate composition. Specimens examined in the present study are deposited in the collection of the School of Applied Bio-sciences, Kyungpook National University (KNU), Daegu, Republic of Korea.

Materials examined. 1 male, KNU campus, Daegu, 5.vi.1982, YJ Kwon; 6 males, same locality, 26.vi.1982; 8 females, same locality, 1.vii.2010; 6 males, 3 females, Hayang Eup, Gyeongsangbuk-do, 14.vii.1984; 1 male, Juwangsan, Gyeongsangbuk-do, 19.vii.1981; 2 females, same locality, 26.vii.1984; 1 female, Naeyeonsan, Gyeongsangbuk-do, 5.vii.1982; 1 male, Ulleungdo, Gyeongsangbuk-do, 18.vi.1983; 23 males, 12 females, Dalsan-myeon, Yeongdeok-gun, Gyeongsangbuk-do, 12.vi.2010; 1 male, Mt.Cheonwhang, Gyeongsangnam-do, 8.vii.1980; 1 female, Hwaaksan, Gyeongsangnam-do, 6.viii.1998; 1 male, Tongdosa Temp., Gyeongsangnam-do, 9.x.1979; 1 male, 2 females, Yongmunsa Temp., Gyeongsangnam-do, 16.vi.1980; 1 female, Hwasun, Jeju-do, 29.v.1992; 2 females, Jungmun, Jeju-do, 12.viii.1984; 5 males, 1 female, Jindo, Jeollanam-do, 17–18.vii.1984; 1 female, Wando, Jeollanam-do, 21.vii.1981; all same collector.

DNA extraction

Adult individuals of *R. iguchii* males (n = 15), *R. iguchii* females (n = 15) and *R. quadricinctus* (n = 15) were separately used for DNA extraction. Total genomic DNA was extracted from a single individual (ethanol-preserved, kept at −20°C) of each category following the manufacturer's instructions with PureLink™ Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA). Immediately before extraction, ethanol-preserved individuals were washed with double-distilled water to remove ethanol and dried onto Whatman 3MM filter papers. The male specimens were homogenized after removal of the aedeagus for morphological observation and species identification.

PCR amplification and gel-electrophoresis

Mitochondrial (COI gene) and ribosomal DNA (ITS2 region) were amplified by polymerase chain reaction (PCR) using each set of primers.

A fragment of the COI mitochondrial gene was amplified using the primers C1-J-2195 (5′-TTGATTTTTTGGTCA TCCAGAAGT-3′) and TL2-N-3014 (5′-TCCAATGC ACTAATCTGCCATATTA-3′) (Simon *et al*. 1994). PCR was performed in 20 µL reaction volume containing 1 × PCR buffer (20 mmol Tris-HCl, pH 8.4, 50 mmol KCl), 1.5 mmol $MgCl_2$, 200 µmol of each deoxynucleotide triphosphate (dNTP), 0.5 µmol of each primer, 1 unit of Taq polymerase (SolGent Co. Ltd, Daegu, Korea) and 87–178 ng of template DNA. The mixtures were amplified in a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA) with 5 min initial denaturation cycle at 94°C, 35 cycles at 94°C for 30 sec, 54°C for 1 min and 72°C for 1 min and a final cycle at 72°C for 10 min (Bertin *et al*. 2010).

The amplification of the ITS2 region was carried out with the primers ITS2-F (5′-TGTGAACTGCAGGAC ACATG-3′) and ITS2-R (5′-ATGCTTAAATTTAGGG GGTA-3′), which respectively anneal on 5.8S and 28S ribosomal regions (Collins & Paskewitz 1996). The PCR conditions were the same as described for COI amplification, except the annealing step (58°C for 45 sec).

Products of both PCR assays were visualized on 1% agarose gel containing ethidium bromide. Expected PCR products were excised from the gel and purified using the Wizard PCR preps DNA purification system (Promega, Madison, WI, USA).

DNA sequence analysis

COI and ITS2 purified product sequences were determined using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed by a 3730XL DNA Sequencer (Applied Biosystems). The mitochondrial and ribosomal sequences of *R. iguchii* (Accession number for COI: JF319660; ITS2: JF319662) and *R. quadricinctus* (Accession number for COI: JF319661; ITS2: JF319663) were deposited in GenBank (National Centre for Biotechnology Information, NCBI). Sequences for *R. quadricinctus* and *R. iguchii* were compared to known sequences in the databases using the BLAST algorithm in NCBI. Sequences were aligned using the ClustalW2 algorithm.

Results

Morphological systematics

Genus *Reptalus* Emeljanov, 1971

Reptalus Emeljanov, 1971: pp. 621–622.
Type-species: *Cixius quinquecostatus* Dufour, 1833.

Subgenus *Reptalus* s. str.

Reptalus s. str. Emeljanov, 1995: pp. 73–89.

Key to species of the subgenus *Reptalus* s. str. from Korea

1. Length of apical process of right genital stylus (Fig. 1l,m) short, wide, and one-third of left stylus (Fig. 1j,k). Forewings of female (Fig. 2d) with scattered dark brown spots on near middle forming a transverse band, and apical half with some scattered dark brown spots. Forewings of male (Fig. 1d) without above color pattern. Body length: 6.2–7.0 mm (male), 7.9–8.2 mm (female) . . . . . ***R. iguchii*** (Matsumura)
– Length of apical process of right genital stylus moderately long, narrow, and two-third of left stylus. Forewings of male and female with four dark brown transverse bands on the base to apex of the wing. Body length: 5.0–5.2 mm (male), 6.0–6.4 mm (female) . . . . . ***R. quadricinctus*** (Matsumura)

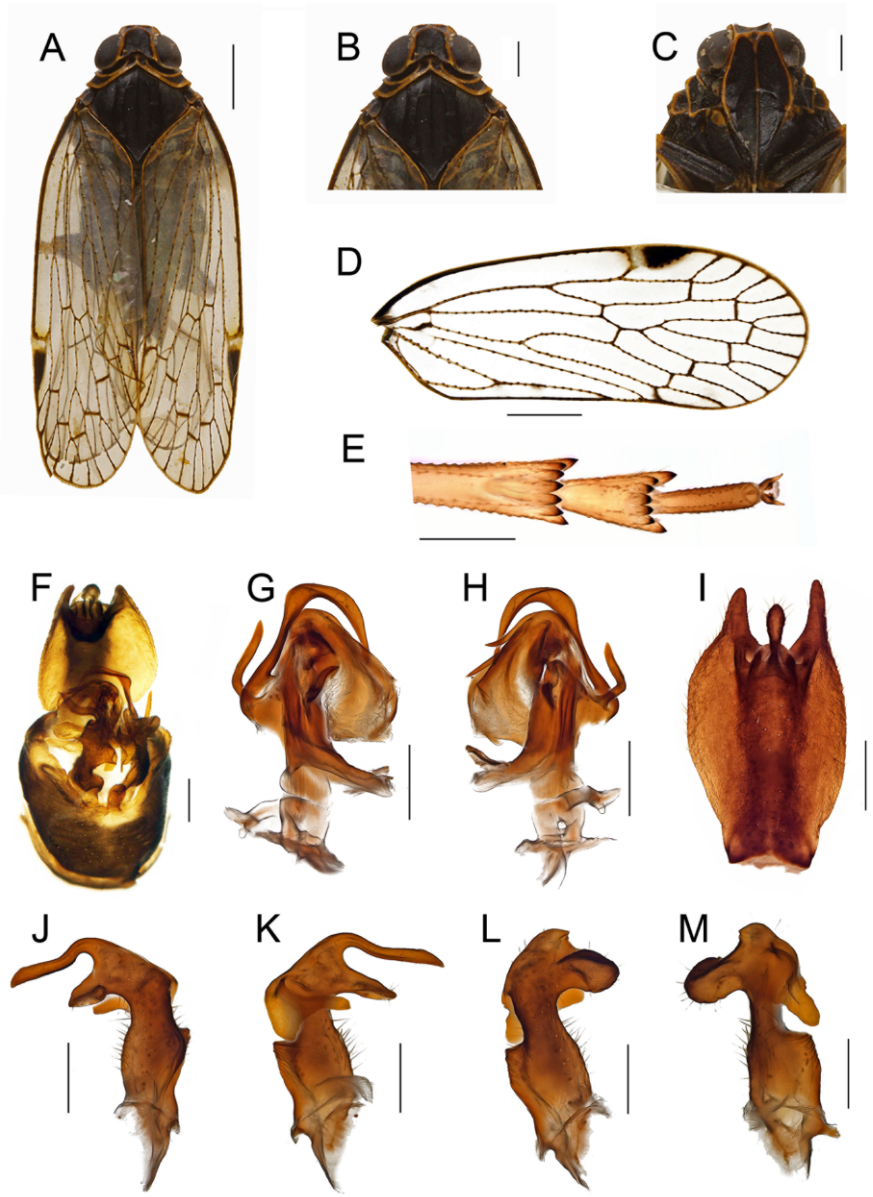

Figure 1 *Reptalus iguchii* (a–m) male structures. (a) Male habitus; (b) head and thorax, dorsal view; (c) frons and clypeus; (d) forewing; (e) 1st, 2nd and 3rd tarsomere of hind leg; (f) male genital block, ventral view; (g) aedeagus, right lateral view; (h) aedeagus, left lateral view; (i) anal segment, dorsal view; (j) left genital stylus, dorso-lateral view; (k) left genital stylus, ventral view; (l) right genital stylus, dorso-lateral view; (m) right genital stylus, ventral view. Scale bars = 1.0 mm (A, D); 0.5 mm (B, C); 0.25 mm (E–I); 0.2 mm (J–M).

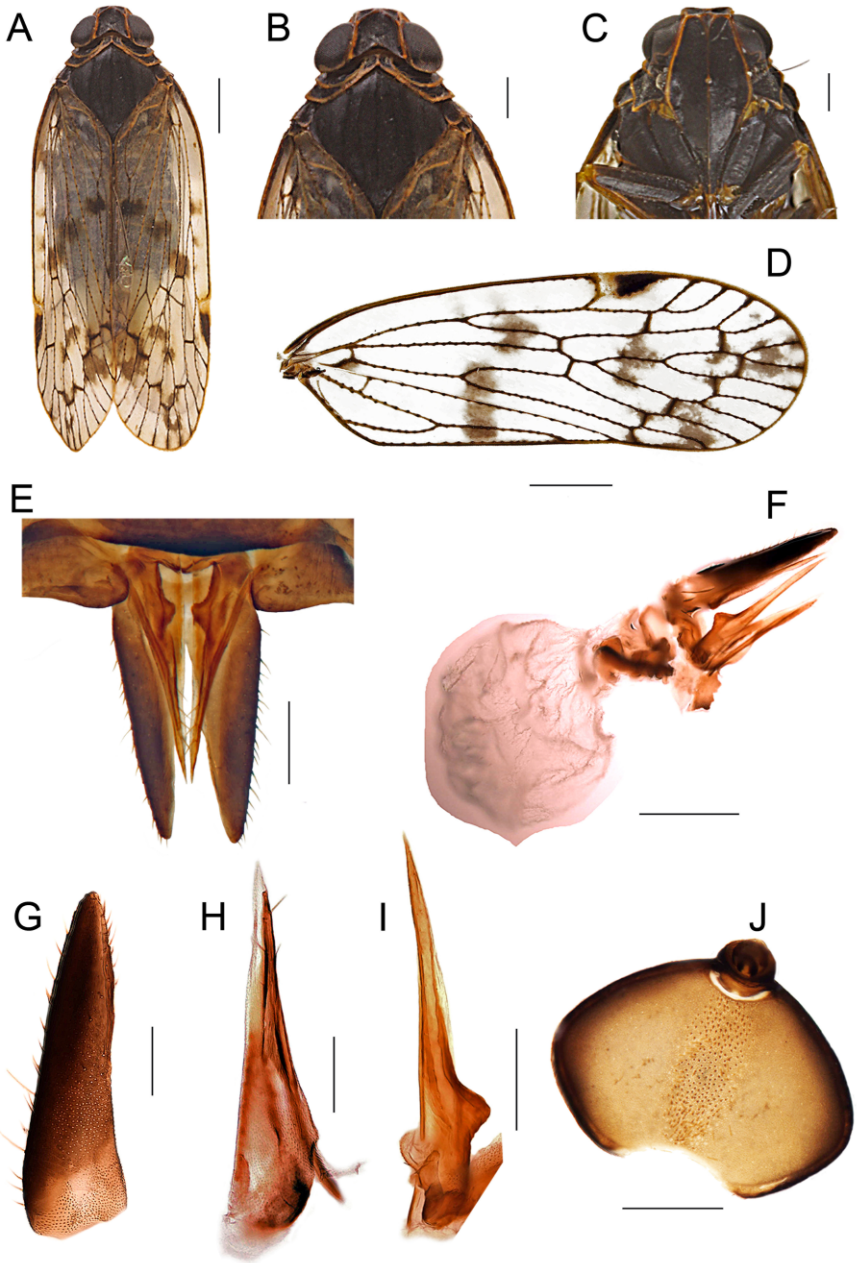

Figure 2 *Reptalus iguchii* (a–j) female structures. (a) Female habitus; (b) head and thorax, dorsal view; (c) frons and clypeus; (d) forewing; (e) ovipositor with gonocoxa, gonoplac and gonapophyses; (f) outer and inner ectodermal genital structures, lateral view; (g) gonoplac, lateral view; (h) gonapophysis VIII, lateral view; (i) gonapophysis IX, lateral view; (j) wax plate with anal segment. Scale bars = 1.0 mm (A, D); 0.5 mm (B, C, F, J); 0.20 mm (E, G, I); 0.1 mm (H).

***Reptalus iguchii* (Matsumura 1914) Comb. nov. (Figs 1,2)**

Oliarus iguchii Matsumura, 1914: p. 419 [Type-locality: Japan]; Nast, 1972: p. 25 (Checklist); Liang & Suwa 1998: p. 140 (Matsumura's type material).

Description of male. Figure 1A–M. Body length (from apex of vertex to tip of forewings): 6.2–7.0 mm (n = 48), Forewing length 5.2–6.0 mm (n = 48).

Coloration. General color brown to dark brown. Vertex (Fig. 1b) black with yellow brown longitudinal and transverse carinae. Frons and clypeus (Fig. 1c) black with yellow

brown lateral and median carinae. Rostrum brown except apex, fuscous. Genae black. Eyes blackish brown, ocelli whitish brown. Antennae brown with yellowish apex. Pronotum (Fig. 1b) black with golden yellow border. Mesonotum (Fig. 1b) black, with blackish five longitudinal carinae, subtriangular margin little yellowish brown. Tegulae roof shaped, black with yellow margin. Forewings (Fig. 1d) hyaline, brownish black pterostigma, transverse veins with dark brown shade, and longitudinal veins yellowish brown with dark brown granules. Hind wings semihyaline with yellow brown veins. Thorax with ventral areas yellow brown to dark brown. Femur black with yellow brown joint, tibia and tarsi brownish. Abdomen blackish brown, except posterior margin of each segment, yellow brown. Genital segment (Fig. 1f) dark brown to brown.

Head and thorax. Head with eyes (Fig. 1b) slightly narrower than pronotum (1:1.15). Vertex as long as wide, with two prominent transverse keels, depressed medially. Frons (Fig. 1c) tricarinated, median carina forked at base. Epistomal suture forms disc shaped post-clypeus, anteclypeus short. Rostrum reaching to hind coxae. Pronotum short with two lobes, collar like. Mesonotum quite large with five distinct longitudinal carinae. Forewings (Fig. 1a,d) longer than wide, more or less flatly folded, and surpassing the tip of the abdomen. First segment of hind tarsus (Fig. 1e) bears all teeth apically, second segment bears 5 teeth with subapical setae (platellae).

Male genitalia. Anal tube (Fig. 1i) flattened, and slightly widened, with sharp asymmetric apical processes, paraproct near to equal of the apical level of two asymmetric processes. Pygofer (Fig. 1f) with asymmetrical laterodorsal processes, ventromedian process well developed, stout, narrow, acute at apex. Genital styli (Fig. 1j–m) complex apically, asymmetric i.e. the right stylus (Fig. 1l,m) not mirror image of left one (Fig. 1j,k), length of right apical process one-third of left one. Distal segment of aedeagus (Fig. 1g–h) with two teeth; theca bears larger right basal tooth with additional tooth at base, and with shorter left tooth.

Redescription of female. Figure 2A–J. Body length (from apex of vertex to tip of forewings): 7.9–8.2 mm (n = 33), Forewing length: 6.9–7.0 mm (n = 33). Coloration. Head and thorax. Similar to male, differing mainly in forewings and size of body parts. Forewings (Fig. 2d) hyaline with scattered dark brown spots on near middle forming a transverse band, and apical half with some scattered dark brown spots, pterostigma brownish black with hairs, transverse veins with dark brown shade, longitudinal veins yellowish brown with dark brown granules. Female genitalia. Outer structure (Fig. 2e,j) brown to dark brown. Ovipositor (Fig. 2e) reduced, waxen field (Fig. 2j) above it well developed with numerous pores, large. Gonoplac (Fig. 2g) dark brown with hairs, apex narrower than the base. Gonapophysis VIII (Fig. 2h) moderately shorter than gonapophysis IX (Fig. 2i), both apical parts bear hair-like setae, inner side of gonapophysis IX little extended near base ventrally, inner structure (Fig. 2f) consists of sac-like bursa copulatrix, diverticulum ductus, ductus receptaculi, oviduct communis hardly visible.

Host. *Artemisia sp.* and *Prunus* sp. as potential host based on collection data.

Distribution. Korea (new record), Japan.

Remarks. The genus *Oliarus* Stål sansu lato was subdivided into more natural smaller genera according to modern standards. The genus *Reptalus* was established including eight *Oliarus* species by Emeljanov in 1971, and subdivided into two subgenera based on the following features (Emeljanov 1995): the genus *Reptalus* differs from other genera belonging to the tribe Pentastirini principally by bearing not more than 6 teeth at apices of each 1st and 2nd segments of hind tarsi with subapical setae (platellae) at least on 2nd segment. The 1st hind tarsal segment bears teeth with subapical setae, and anal tube weakly asymmetric without sharp processes near apex in the subgenus *Trepalus*. On 1st hind tarsal

Figure 3 Male and female of *Reptalus iguchii* in copulation (female above, male below).

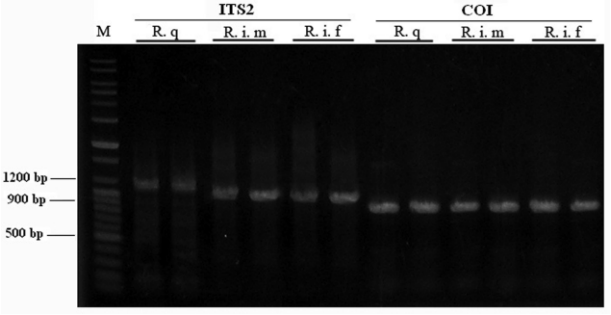

Figure 4 Agarose gel electrophoresis of internal transcribed spacer 2 (ITS2) and cytochrome oxidase subunit I (COI) polymerase chain reaction products from R.q., *Reptalus quadricinctus*; R.i.m., *Reptalus iguchii* male; R.i.f., *Reptalus iguchii* female; M, molecular weight marker (100 bp ladder).

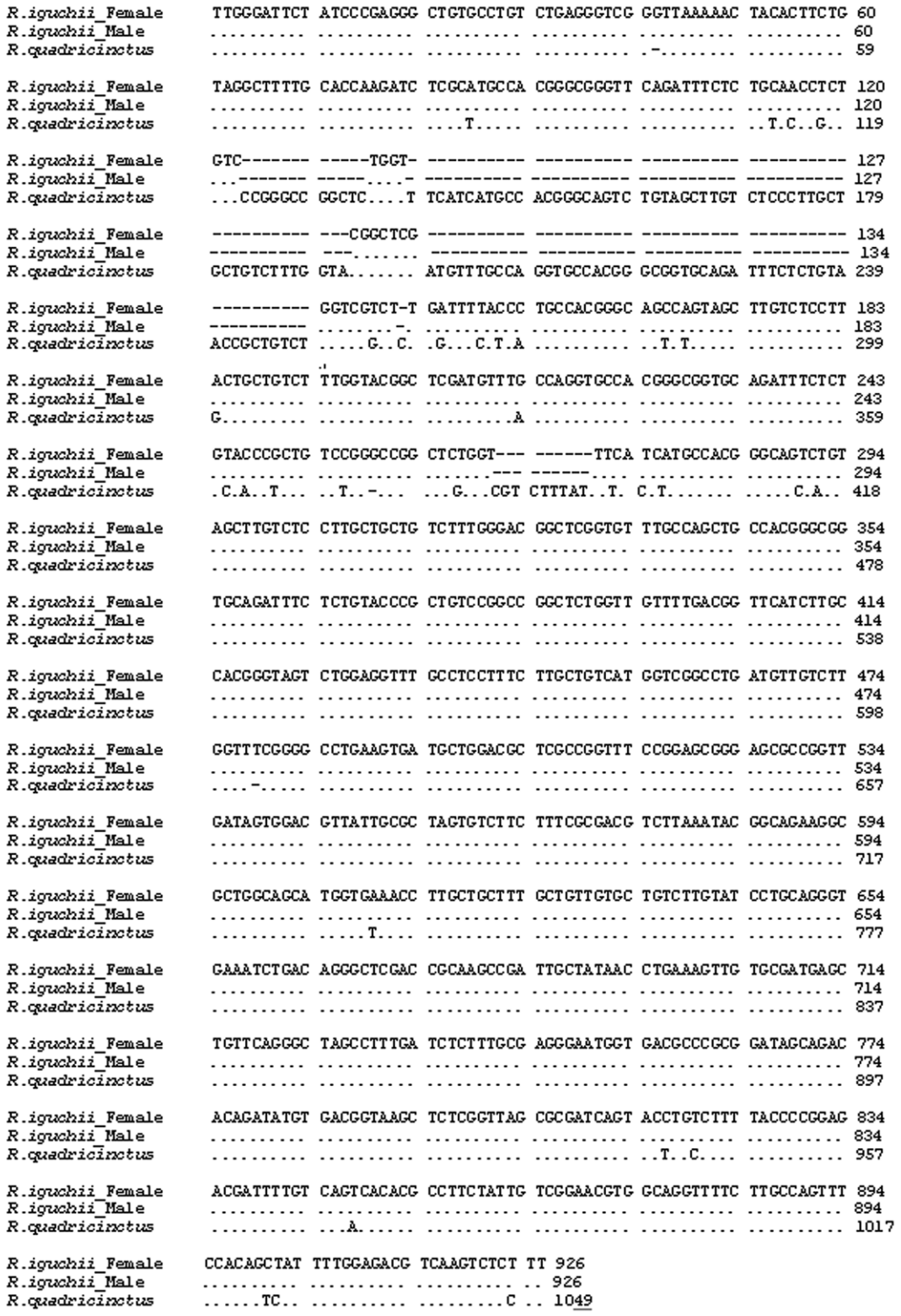

Figure 5 Internal transcribed spacer 2 (ITS2) partial sequence (5′–3′) of *Reptalus iguchii* female and male (JF319662) compared with the sequence of *Reptalus quadricinctus* (JF 319663). Identities with the first sequence are indicated by dots (.); dashes (-) represent gaps.

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R.iguchii_Female    CAGGATTTGG ATTAATTTCT CATATTATTA TAAAAGAAAG AGGAAAAAAA GAAACATTTG 60
R.iguchii_male      .......... .......... .......... .......... .......... .......... 60
R.quadricinctus     .......... .......... .......... .......... .......... ..........  60

R.iguchii_Female    GATCAATCGG AATAATTTAT GCAATAATTG CTATTGGAAT CCTAGGATTT GTAGTATGAG 120
R.iguchii_male      .......... .......... .......... .......... .......... ..........  120
R.quadricinctus     .......... .......... .......... .......... .......... ..........  120

R.iguchii_Female    CTCACCATAT ATTTACAGTC GGAATAGATA TTGATACACG AGCTTATTTT ACATCAGCTA 180
R.iguchii_male      .......... .......... .......... .......... .......... ..........  180
R.quadricinctus     ....T..... .......... .......... .......... .......... ..........  180

R.iguchii_Female    CAATAATTAT TGCAGTTCCA ACAGGAATTA AAATTTTCAG ATGAATAGCA ACAATTTATG 240
R.iguchii_male      .......... .......... .......... .......... .......... ..........  240
R.quadricinctus     .......... .......... .......... .......... .......... ..........  240

R.iguchii_Female    GAACAAAAAT TAAATTTACA CCACAAACTA TATGAGCAAT GGGATTCATT TTTTTATTTA 300
R.iguchii_male      .......... .......... .......... .......... .......... ..........  300
R.quadricinctus     .......... .......... .......... .......... A.....T... ..........  300

R.iguchii_Female    CCATAGGAGG ATTAACAGGA GTAATTCTTG CAAACTCATC AATTGATATT ATTTTGCACG 360
R.iguchii_male      .......... .......... .......... .......... .......... ..........  360
R.quadricinctus     .......... .......... .......... ....T..... .......... ..........  360

R.iguchii_Female    ATACATATTA TGTAGTAGCT CATTTCCATT ACGTTTTATC AATAGGAGCA GTATTTGCTA 420
R.iguchii_male      .......... .......... .......... .......... .......... ..........  420
R.quadricinctus     .......... .......... .......... .......... .......... ..........  420

R.iguchii_Female    TTATAGGAAG ATTTATTGAA TGATACCCAT TAATAACAGG CTTATCAATA AACCAAAAAT 480
R.iguchii_male      .......... .......... .......... .......... .......... ..........  480
R.quadricinctus     .......... .......... .......... .......... .......... ..........  480

R.iguchii_Female    GACTAAAAAT TCAATTCCTT ACTATATTTA CAGGAGTAAA TTTAACATTT TTCCCTCAAC 540
R.iguchii_male      .......... .......... .......... .......... .......... ..........  540
R.quadricinctus     .......... .......... .......... .......... ......G... ........G.  540

R.iguchii_Female    ACTTCTTAGG ACTATCTGGT ATACCACGAC GATATTCTGA TTATCCAGAT ACATACATAT 600
R.iguchii_male      .......... .......... .......... .......... .......... ..........  600
R.quadricinctus     ....T..... .......... .......... ....C..... .......... .....T....  600

R.iguchii_Female    CATGAAATAT AATTTCATCT ATAGGTTCAA TAATTTCTTT ATTAAGAATT ATAATAATAA 660
R.iguchii_male      .......... .......... .......... .......... .......... ..........  660
R.quadricinctus     .......C.. .......... .......... ........A. .......... ..........  660

R.iguchii_Female    TATTTATCCT TTGAGAAAGA ATAACTTTTA AACGAACAAT CTTATTTAAA AATAATAGTT 720
R.iguchii_male      .......... .......... .......... .......... .......... ..........  720
R.quadricinctus     .......... C......... .......... .......... .......... ..........  720

R.iguchii_Female    CTTCATCACT AGAGTGAGAA TTAAATAATC CTCCAGCAGA GCACTGCTTT AATGAATTAC 780
R.iguchii_male      .......... .......... .......... .......... .......... ..........  780
R.quadricinctus     .......G.. .......... .......... .......... .......... ..........  780
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Figure 6 Cytochrome oxidase subunit I (COI) partial sequence (5′–3′) of *Reptalus iguchii* female and male (JF319660) compared with the sequence of *Reptalus quadricinctus* (JF319661). Identification with the first sequence are indicated by dots.

segment all teeth without subapical setae, and anal tube usually with sharp asymmetric apical processes in the subgenus *Reptalus* s. str. (Holzinger *et al*. 2003; Wilson 2005; Bertin *et al*. 2010). The poorly known species, *Oliarus iguchii*, Matsumura 1914, bears all representative characters of the genus *Reptalus* and subgenus *Reptalus* s.str. Therefore, this species is in need of placement in the subgenus *Reptalus* s. str. within the genus *Reptalus*.

The previously mentioned female specimens were compared with the color image of Matsumura's specimen deposited in Hokkaido University Museum Database (Hokkaido University 2007) and with the original description of Matsumura (1914).

Copulation based on a pair preserved *in copula*

A pair of *R. iguchii in copula* (Fig. 3) was collected during field work. During copulation, hooks of male genital styli, anal tube and pygofer all assist in holding the partners together. Some twisting of the male abdomen is needed during copulation because of the upward position of female genitalia. Such possible twisting may be lost when joined specimens are killed (Sforza & Bourgoin 1998; Wang *et al*. 2009). Sexual dimorphism in wing color pattern is confirmed by the association of the sexes.

ITS2 sequence: PCR analysis

The ITS2 region was successfully amplified from each of the males and females of *R. iguchii* and *R. quadricinctus* providing fragments with the following sizes: 1180 bp for *R. quadricinctus*; 1020 bp for *R. iguchii* (male); 1020 bp for *R. iguchii* (female) (Fig. 4). The male and female of *R. iguchii* shared an identical sequence, but the *R. quadricinctus* differed from that of *R. iguchii* (similarity score between male and female of *R. iguchii* using ClustalW2: 100%, but among *R. iguchi* and *R. quadricinctus*: 96%) (Fig. 5).

CO1 sequence: PCR analysis

Amplification of the mitochondrial COI gene from all tested individuals always provided a 920 bp fragment (Fig. 4). The male and female of *R. iguchii* shared an identical sequence, but the sequence of *R. quadricinctus* differed from that of *R. iguchii* (similarity score between male and female of *R. iguchii* using ClustalW2: 100%, but among *R. iguchii* and *R. quadricinctus*: 98%) (Fig. 6).

Discussion

This study provides a comprehensive description of both morphological and molecular tools for identification of a sexually dimorphic species, *R. iguchii*. This species is reported for the first time in Korea. Previously, it was described based on a single female specimen, whereas description of males is needed for recognition of species of this subgenus. In this paper, a morphological description of the male is provided for the first time with illustrations (Fig. 1). The taxonomic position of this species is noted in the remarks in the Result section. A key to species is given for distinguishing males and females of this species, and separating them from the other species of this genus from Korea.

Two different DNA regions, ribosomal ITS2 and mitochondrial COI, were sequenced to test species concepts. Both molecular markers provide evidence that the analyzed males belong to the same species as associated females. Sequence alignment similarity score between males and females (100%) led to the conclusion that the collected males represented *R. iguchii*. Both markers also exclude possible misidentification of this species with *R. quadricinctus*. Although COI-PCR provided a 920 bp fragment for all tested individuals, the alignment of COI sequences revealed a certain degree of dissimilarity among two species, *R. iguchii* and *R. quadricinctus*. In fact, ITS2-PCR allowed us to recognize two species directly by amplicon size (*R. iguchii*: 1020 bp, *R. quadricinctus*: 1180 bp). Therefore, we concluded that the ITS2 region provides the simplest tool for rapid identification of these closely related species by PCR assay.

Only adults of *R. iguchii* are collected from *Prunus* and *Artemisia* plants, which are recognized herein as potential hosts of that species. However, further intensive investigations are needed on the above-mentioned plants to confirm their status as host plants.

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