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3 **Genetic differentiation of *Ganaspis brasiliensis* (Hymenoptera: Figitidae) from East**
4 **and Southeast Asia**

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Abstract

Ganaspis brasiliensis (Ihering) (Hymenoptera: Figitidae: Eucoilinae) is a *Drosophila* parasitoid that has often been misidentified as *G. xanthopoda* (Ashmead) in recent studies. This study aims to clarify genetic differentiation of *G. brasiliensis* based on the nucleotide sequences of the mitochondrial *cytochrome oxidase subunit 1* (*CO1*) gene and three nuclear DNA regions, the inter-transcribed spacers 1 and 2 (ITS1 and ITS2) and putative 60S ribosomal protein L37 (*RpL37*), as well as crossing experiments. Four lineages are recognized in individuals assigned as *G. brasiliensis* by morphology, 1) individuals occurring in Japan and probably South Korea, 2) individuals from a small subtropical island of Japan, Iriomote-jima, 3) individuals from temperate lowlands of Japan and high altitude areas of Southeast Asia, and 4) individuals occurring widely in Asia, America, Hawaii and Africa. The first lineage is a specialist of *Drosophila suzukii* (Matsumura), a pest of fresh fruit, and also the fourth lineage has a capacity to parasitize this pest species. The first, third and fourth lineages occur sympatrically at least in Tokyo. The third and fourth lineages differed in mate choice and host use to some extent, but post-mating isolation between them was almost absent.

Keywords *Drosophila suzukii* • Nucleotide sequence • Parasitoids • Reproductive isolation • Species status

Introduction

Drosophila suzukii (Matsumura) is a fruit crop pest causing serious economic loss in Asia, Europe and North America (Asplen et al. 2015; Kanzawa 1939). To reduce fruit crop damages by this pest, the development of a biological control program is desired, as current measures, such as insecticide application, or net covering, incur some environmental loads and economic costs. So far, *Ganaspis xanthopoda* (Ashmead) (Hymenoptera: Figitidae) has been reported as a major parasitoid attacking *D. suzukii* in central Japan (Kasuya et al. 2013b). However, there are a number of questions on this parasitoid species including its species identification. *Ganaspis xanthopoda* was described from Grenada in Lesser Antilles in the Caribbean Sea (Ashmead 1896), and now it has been widely recorded from North America, South America, Hawaii, Asia and Africa (Ashmead 1896; Carton et al. 1986; Kimura and Suwito 2012, 2015; Mitsui and Kimura 2010; Mitsui et al. 2007; Schilthuizen et al. 1998). From Japan, two types have been known in this species; i.e., the *suzukii*-associated type and the *lutescens*-associated type parasitizing *Drosophila lutescens* Okada and some other *Drosophila* species breeding on fermenting fruits, which also differ in the nucleotide sequences of the mitochondrial *cytochrome oxidase subunit 1* (*CO1*) gene and the inter-transcribed spacer 1 and 2 (ITS1 and ITS2), although they show only small differences in morphology (Kasuya et al. 2013b; Mitsui and Kimura 2010). However, Buffington and Forshage (2016) and Daane et al. (2016) recently reported that a *Ganaspis* species parasitizing *D. suzukii* in South Korea is *Ganaspis brasiliensis* (Ihering), which was

described from Brazil. To solve this inconsistency, we have reexamined the morphology of *Ganaspis* individuals collected from Japan. As a result, *Ganaspis* individuals so far assigned as *G. xanthopoda* in our previous papers (Kasuya et al. 2013b; Mitsui and Kimura 2010; Mitsui et al. 2007) are determined as *G. brasiliensis*, and *Ganaspis* sp. TK2 reported by Kasuya et al. (2013a) is determined as *G. xanthopoda*.

In *G. brasiliensis*, in addition to the *suzukii*- and *lutescens*-associated types referred above, Schilthuizen et al. (1998) reported some individuals from Thailand and Philippines (assigned as *G. xanthopoda*), which differ from these two types to some extent in the nucleotide sequences of ITS1 and ITS2. In addition, the nucleotide sequences of the *CO1* gene of specimens from Uganda and Hawaii that are registered in NCBI database as *G. xanthopoda* differ from that of the two types to some extent. Thus, there seems to be much variation in *G. brasiliensis*. It is therefore important to clarify the genetic diversity and species status of this species to use it as an agent for biological control of *D. suzukii*. In this study, we investigate the phylogeny and species status of East and Southeast Asian specimens of *Ganaspis* species by molecular phylogenetic analyses based on the nucleotide sequences of the mitochondrial *CO1* gene and three nuclear DNA regions, ITS1, ITS2 and a putative *60S ribosomal protein L37 (RpL37)* gene. In addition, we conducted cross experiments to examine reproductive isolation between three strains of *G. brasiliensis* collected from Taiwan and Japan.

Materials and methods

Samples

Individual *Ganaspis* specimens used for molecular phylogenetic analysis were obtained from Bogor and Cibodas in Indonesia, Kinabalu in Malaysia, Kaohsiung in Taiwan, and Iriomote-jima, Kagoshima, Tokyo, Sendai and Sapporo in Japan (Table 1, Fig. 1). The specimens were reserved in Hokkaido University Museum. In addition, laboratory strains of *G. brasiliensis* were established with specimens collected from Kaohsiung (KS) in March 2009, Tokyo (TK) in May 2006, and Sapporo (SP) in August 2013, to investigate reproductive isolation. The KS and SP strains were reared using *Drosophila simulans* Sturtevant as host, and the TK strain was reared using *D. lutescens* as host. These strains were maintained under a long daylength (15 h light:9 h dark) at 23 °C for several years before experiments.

Molecular methods

DNA was extracted from samples using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Target fragments were amplified by polymerase chain reaction (PCR). For *CO1*, two separate regions were amplified with the following primer pairs, LCO/HCO (LCO: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3', 440–688 bp; Folmer et al. 1994) and hco-extA/hco-extB (hco-extA: 5'- GAAGTTTATATTTTAATTTTACCTGG-3' and hco-extB: 5'-CCTATTGAWARAACATARTGAAAATG-3', 326–376 bp; Schulmeister

et al. 2002). ITS1, ITS2, and *RpL37* fragments were amplified using primer pairs, 7246/7247, I-2a/I-2b, and 27F/27R (Lohse et al. 2010), respectively (7246: 5'-GCTGCGTTCTTCATCGAC-3' and 7247: 5'-CGTAACAAGGTTTCCGTAGG-3', 241–736 bp; I-2a: 5'-TGTCAACTGCAGGACACATG-3' and I-2a: 5'-AATGCTTAAATTTAGGGGGTA-3', 239–531 bp; 27F: 5'-GAARGGTACNTCVAGYTTTGG-3', 27R: 5'-GACCRGTDCCRGTRGTCTTCCT-3', 520–766 bp). For samples that did not amplify with 27F/27R, a reverse primer, 7g2r (TGCTWATTTCTACTTATTTCAATTGCT), was developed using Primer3 (Untergasser et al. 2012) and paired with 27F. The reaction was performed in a mixture containing 1.0 µl sample DNA, 2.0 µl 10× buffer, 2.5 mM MgCl₂, 100 µM dNTP, 0.5 µM each primer and 0.5 U AmpliTaq DNA polymerase (Applied Biosystems) in total volume of 20 µl. The thermal profile for *CO1*, ITS1 and ITS2 consisted of 94 °C for 10 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min, followed by final extension at 72 °C for 1.5 min. *RpL37* was amplified with a touch down PCR consisting of 94 °C for 3 min, 10 cycles of 94 °C for 15 s, 60–50 °C for 40 s, and 72 °C for 1.0 min, 30 cycles of 94 °C for 15 s, 51 °C for 40 s and 72 °C for 1.0 min, followed by 72 °C for 10 min. Prism BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems) was used for sequence reactions. Sequencing was conducted with an ABI 3100 automated sequencer.

Phylogenetic analysis

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141 All the sequences were aligned and adjusted by eye in MEGA 5.2 (Tamura et al. 2011).
142 Substitution models were chosen with BIC (Bayesian information criterion) calculated
143 by jModelTest (Darriba et al. 2012). The model for *COI* sequences was GTR + Γ . ITS1,
144 ITS2, and *RpL37* fragments were concatenated to estimate a single nDNA tree, with
145 separate substitution models (HKY+ Γ , HKY+I, and HKY+ Γ , respectively). Rate
146 variation across branches was assumed to follow exponential distribution (relaxed-clock
147 model with uncorrelated rates; Drummond et al. 2006), and it was validated against
148 strict-clock (Bayes factor for *COI* = 34.23 ; nDNA = 22.01) (Baele and Lemey 2013;
149 Kass and Raftery 1995). Models were fitted using BEAST2 (Bouckaert et al. 2014) and
150 the convergence was confirmed in Tracer (Rambaut et al. 2014). Trees were visualized
151 using FigTree (Rambaut 2014). The sequences of *G. brasiliensis* (assigned as *G.*
152 *xanthopoda*) from other locations (Hawaii, Philippines, Thailand, and Uganda) were
153 obtained from NCBI database and used for the reconstruction of phylogenetic trees.

154 In addition, genetic distances between *COI* sequences were calculated with
155 Kimura's two-parameter model and pairwise deletion using R package "APE" (Paradis
156 et al. 2004) in R 3.2.2 (R Development Core Team 2015).

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158 Crossing experiments

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160 The level of reproductive isolation among the KS, TK and SP strains was examined by
161 cross experiments. Virgin females used for cross experiments were obtained by rearing

host puparia individually in separate small vials. Five virgin females and five males from each strain were placed together in a vial with *Drosophila* medium (cornmeal 50 g, wheat germ 50 g, sugar 50 g, dry yeast 40 g and propionic acid 5 ml in 1000 ml of water) for mating for a day, and then they were transferred to a vial containing approximately 300 two-day old *D. melanogaster* (the Harwich strain) larvae. In the cross between females and males from the same strains, five females and males were collected directly from the original stock and placed in a vial containing *Drosophila* medium and *D. melanogaster* larvae. When F₁ parasitoids emerged, they were collected and examined for the sex ratio (proportion of females). Because this species is arrhenotokous as in most other hymenopteran species, unmated females produce male progenies, whereas females mated with conspecific males usually produce both female and male progenies. Therefore, the proportion of females in progenies suggests how frequently sperm is used in the production of progenies; i.e. it can be used as an indicator of reproductive isolation. If both F₁ males and females emerged, two to five F₁ individuals of each sex were placed in a new vial with host larvae and allowed to reproduce. In the same way, the production and sex ratio of F₂ and F₃ were examined. Four replicates were prepared for each cross. Experiments were conducted under a long daylength (15:9 h light:dark) at 23 °C. For progenies, deviation from the 1:1 sex ratio was examined with χ^2 test with sequential Bonferroni correction using Jmp ver 6.1 (SAS Institute, Cary, USA).

Results

Phylogenetic analysis

Figure 2 shows a tree based on the nucleotide sequences of the *CO1* region, and Fig. 3 shows a tree based on concatenated ITS1, ITS2 and *RpL37* sequences. Both trees revealed that *G. brasiliensis* and *G. xanthopoda* were distantly related among *Ganaspis* species studied here. In the *CO1* tree, individuals morphologically identified as *G. brasiliensis* can be subdivided into five groups, 1) individuals parasitizing *D. suzukii*, 2) those from Iriomote-jima (IR), 3) those from temperate lowlands of Japan (TK, SD: Sendai, KG: Kagoshima) and high-altitude areas of tropical regions (CB: Cibodas, KB: Mt. Kinabalu), 4) those from Indonesia (BG: Bogor), and 5) those from Japan (TK: Tokyo and SP: Sapporo), Taiwan (KS: Kaohsiung), Uganda (UG) and Hawaii (HW). The last group can be further subdivided into two subgroups; i.e., those from Japan (TK and SP) and Taiwan (KS) and those from UG and HW. The nDNA tree agrees well with the *CO1* tree, except that the groups 4 and 5 in the *CO1* tree are not clearly discriminated. Individuals from the Philippines and Thailand are included in a complex of the groups 4 and 5.

For the *CO1* sequences, genetic distances between individuals of the 5 groups were calculated with Kimura's two-parameter model and pairwise deletion. The genetic distances between individuals of group 1 and those of groups 2, 3, 4 and 5 ranged from 0.047 to 0.071, and the distances between individuals of group 2 and those of groups 3, 4 and 5 ranged from 0.031 to 0.043. On the other hand, the distances between

individuals of 3, 4, and 5 groups ranged from 0.013 to 0.025.

Crossing experiments

Table 2 shows the results of cross experiments. The sex ratio was significantly deviated from 1:1 in all cases where progenies were obtained (χ^2 test with sequential Bonferroni correction, $p < 0.05$). In the crosses between females and males from the same strains, the sex ratio F_1 offspring was male biased. In the cross between KS and SP, the sex ratio of F_1 offspring was also male biased, but the sex ratio of F_2 and F_3 offspring was closer to 1:1. In the cross between KS or SP females and TK males, almost only male offspring was obtained, probably because mating did not occur. In the cross between SP females and TK males, F_2 and F_3 offspring were obtained, and their sex ratio was closer to 1:1 than F_1 offspring. In the cross between TK females and KS or SP males, F_1 offspring were produced, but their number was not large in comparison with other crosses. In these crosses, the sex ratio was male biased in F_1 and F_2 offspring, but closer to 1:1 in F_3 offspring.

Discussion

Ganaspis brasiliensis has often been misidentified as *G. xanthopoda*, but these two species are clearly distinctive not only morphologically but also genetically. In the present molecular study, individuals reported as *G. xanthopoda* by Schilthuizen et al.

(1998) are revealed as *G. brasiliensis* as well as those reported by Mitsui et al. (2007), Mitsui and Kimura (2010), Kasuya et al. (2013b) and Kimura and Suwito (2012, 2015). Other *Ganaspis* individuals so far assigned as *G. xanthopoda* by *Drosophila* researchers (e.g., Carton et al. 1986) would also be *G. brasiliensis*.

As a consensus of the *CO1* and nDNA trees, individuals identified as *G. brasiliensis* by morphology were subdivided into four lineages; 1) individuals associated with *D. suzukii*, 2) individuals from Iriomote-jima, 3) individuals from temperate areas of Japan and high altitude areas of Southeast Asia, and 4) individuals occurring in Asia, Hawaii and Africa. All the four lineages are recorded from Asia, suggesting that their common ancestor occurred in Asia.

The first lineage is a specialist of *D. suzukii* and was previously assigned as the *suzukii*-associated type of *G. xanthopoda* by Kasuya et al. (2013b). This lineage has so far been recorded from Japan (Kasuya et al. 2013b; Mitsui et al. 2007), and individuals reported by Buffington and Forshage (2016) from South Korea would also belong to this lineage. This lineage is expected to have wider distributions, because *D. suzukii* is distributed not only in Japan but also in China, Southeast Asia and India (Lemeunier et al. 1986). This lineage is assumed as a specialist of *D. suzukii* (Kasuya et al. 2013b).

The second lineage has so far been recorded only from Iriomote-jima, an island located at the southern end of the Ryukyu archipelago. However, few studies have been conducted on *Drosophila* parasitoids in the Ryukyu archipelago and also in west Pacific islands. Further sampling is needed in these regions.

The third lineage is a generalist; it mainly parasitizes *Drosophila lutescens*, *D. rufa* Kikkawa & Peng and *D. biauraria* Bock & Wheeler in Japan (Mitsui and Kimura 2010) and previously assigned as the *lutescens*-associated type of *G. xanthopoda* by Kasuya et al. (2013b). Females of this lineage do not oviposit in *D. suzukii* larvae (Mitsui and Kimura 2010). The geographic distribution of this lineage is unique; it occurs in tropical highlands and temperate lowlands (Kimura and Suwito 2015; Mitsui and Kimura 2010). Interestingly, a similar pattern of distributions is known for its host *Drosophila* species, although its hosts in temperate lowlands and tropical highlands are not conspecific; i.e., *D. lutescens* is distributed in temperate lowlands of Asia whereas its close relatives such as *Drosophila* sp. aff. *takahashii* and *D. trilineata* are distributed in tropical and subtropical highlands, and *D. rufa* and *D. biauraria* occur in temperate lowlands whereas their relative *D. trapezifrons* Okada occurs in subtropical highlands (Goto et al. 2000; Kimura and Suwito 2015; Kimura et al. 1994). This suggests a possibility that this lineage of *G. brasiliensis* has expanded the distribution corresponding to the distributions of host species.

The fourth lineage shows a world-wide distribution (Asia, Hawaii and Africa). Organisms that show such wide distributions are often associated with humans. For example, *Drosophila* species that show such world-wide distributions inhabit domestic environments (Dobzhanski 1965). However, it is unclear whether this lineage of *G. brasiliensis* is associated with humans or not. The type specimen of *G. brasiliensis* that was collected in Brazil is assumed to belong to this lineage because the other lineages have not been recorded outside of Asia. It is noticeable that three clades are recognized

in this lineage in the *CO1* tree; i.e., individuals from Indonesia (BG), those from Uganda and Hawaii, and those from Japan and Taiwan. Geographic differentiation may have occurred to some extent in this lineage. This lineage would be a generalist parasitizing a number of *Drosophila* species (Kimura and Suwito 2012), and at least individuals from Hawaii and Uganda have a capacity to parasitize *D. suzukii* (Kacsoh and Schlenke 2012).

The cross experiments suggest that there is no reproductive isolation between the KS and SP strains of the fourth lineages. On the other hand, it is assumed that mating seldom occurred between females of the KS and SP strains and males of the TK strain of the third lineage, although mating occurred more frequently in the reciprocal cross. Thus, there would be some premating isolation between the third and fourth lineages. However, there seems to be no postmating isolation between them, because F₂ and F₃ offspring were abundantly produced in the crosses between the TK and KS or SP strains. These lineages also differ in host use; the KS and SP strains successfully parasitized *D. simulans* (Kimura personal observation), but the TK strain showed low viability in this *Drosophila* species (Mitsui and Kimura 2010). However, parasitism of *D. simulans* by *G. brasiliensis* has been rarely reported in nature (Kimura 2015; Mitsui and Kimura 2010; Mitsui et al. 2007), although *D. simulans* are abundant in Japan.

The present and previous studies (Kasuya et al. 2013b) suggest that the *suzukii*-associated type of *G. brasiliensis* could be used as an agent for biological control and integrated managements of *D. suzukii* and this type is discriminated from the other lineages by the nucleotide sequences of *CO1*, ITS1, ITS2 and *RpL37*. At

present, no definite morphological difference has been found between these lineages (Kasuya et al. 2013b).

It is noteworthy that the three lineages coexisted at least in Tokyo. If genetically differentiated populations are present sympatrically, they are generally recognized as different species. However, reproductive isolation between lineages 3 and 4 is incomplete, and the genetic distance between them was not high (0.013–0.025). As mentioned before, lineage 4 may be an invasive species and may have recently colonized Japan. If this is the case, it is worth investigating whether these two lineages fuse upon hybridization or continue differentiation sympatrically. On the other hand, the *suzukii*-associated type (lineage 1) differed 4–5 % from the other lineages in the *CO1* sequences, suggesting a possibility that it has differentiated from the others at species level. To determine the species status of this type, it is needed to conduct mating experiments.

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Table 1 Accession numbers of sequence fragments derived from specimens sequenced in this study

Samples	Locality	CO1 LCO/HCO	CO1 hco-extA/hco-extB	ITS1	ITS2	RpL37
<i>Ganaspis</i> sp. IR1 <i>Drosophila daruma</i>	Iriomote-jima	LC122439	LC122050	LC120769	LC122341	–
<i>Ganaspis</i> sp. IR2 <i>Drosophila albomicans</i>	Iriomote-jima	LC122438	LC122051	LC120768	LC122340	–
<i>Ganaspis</i> sp. TK1 <i>Scaptodrosophila coracina</i>	Tokyo	AB624299	AB624311	LC120756	LC122331	–
<i>Ganaspis xanthopoda</i> <i>Drosophila bizonata</i>	Tokyo	AB624300	AB624312	LC120755	LC122330	LC122580
<i>Ganaspis brasiliensis</i> <i>Drosophila eugracilis</i> (1)	Bogor	LC122447	LC122025	(LC120757)	–	(LC122581)
<i>Ganaspis brasiliensis</i> <i>Drosophila eugracilis</i> (2)	Bogor	LC122448	LC122026	LC120758	LC122334	LC122569
<i>Ganaspis brasiliensis</i> <i>Drosophila ficusphila</i> (1)	Iriomote-jima	LC122441	LC122027	LC120760	LC122333	LC122570
<i>Ganaspis brasiliensis</i> <i>Drosophila ficusphila</i> (2)	Iriomote-jima	LC122440	LC122028	LC120759	LC122332	LC122571
<i>Ganaspis brasiliensis</i> <i>Drosophila lutescens</i> (6)	Sendai	–	–	–	AB678763	–
<i>Ganaspis brasiliensis</i> <i>Drosophila lutescens</i> (7)	Sendai	–	–	–	AB678764	–
<i>Ganaspis brasiliensis</i> <i>Drosophila lutescens</i> (22)	Tokyo	LC122453	LC122032	AB678754	AB678769	LC122574
<i>Ganaspis brasiliensis</i> <i>Drosophila lutescens</i> (23)	Tokyo	LC122454	LC122033	AB678755	AB678770	LC122575
<i>Ganaspis brasiliensis</i> <i>Drosophila</i> sp. aff. <i>takahashii</i> (1)	Cibodas	LC122437	LC122034	LC120763	LC122335	LC122562
<i>Ganaspis brasiliensis</i> <i>Drosophila</i> sp. aff. <i>takahashii</i> (2)	Cibodas	LC122444	LC122035	(LC120764)	–	(LC122560)
<i>Ganaspis brasiliensis</i> <i>Drosophila suzukii</i> (1)	Sendai	AB678734	LC122038	LC120761	AB678771	LC122565
<i>Ganaspis brasiliensis</i> <i>Drosophila suzukii</i> (2)	Tokyo	AB678735	LC122039	–	–	(LC122572)
<i>Ganaspis brasiliensis</i> <i>Drosophila suzukii</i> (3)	Tokyo	AB678736	LC122040	AB678757	AB678772	LC122566
<i>Ganaspis brasiliensis</i> <i>Drosophila suzukii</i> (4)	Tokyo	AB678737	LC122045	(AB678758)	(LC122343)	–
<i>Ganaspis brasiliensis</i> <i>Drosophila suzukii</i> (5)	Tokyo	AB678738	LC122046	AB678759	AB678773	LC122573
<i>Ganaspis brasiliensis</i> <i>Drosophila suzukii</i> (6)	Tokyo	AB678739	LC122047	AB678760	AB678774	LC122568

<i>Ganaspis brasiliensis</i> unknown host (1)	Kaohsiung	LC122443	LC122042	LC120766	LC122346	—
<i>Ganaspis brasiliensis</i> unknown host (2)	Kaohsiung	LC122455	LC122043	LC120762	LC122336	LC122576
<i>Ganaspis brasiliensis</i> unknown host (3)	Mt. Kinabalu	LC122449	LC122044	LC120765	LC122342	LC122577
<i>Ganaspis brasiliensis</i> unknown host (4)	Tokyo	AB456710	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (5)	Sendai	AB456711	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (6)	Kagoshima	LC122456	LC122052	LC120771	LC122347	LC122583
<i>Ganaspis brasiliensis</i> unknown host (7)	Kagoshima	LC122457	LC122053	LC120772	LC122348	LC122582
<i>Ganaspis brasiliensis</i> unknown host (8)	Sapporo	LC199282	LC199285	LC199291	LC199288	LC199293
<i>Ganaspis brasiliensis</i> unknown host (9)	Sapporo	LC199283	LC199286	LC199292	LC199289	LC199294
<i>Ganaspis brasiliensis</i> unknown host (10)	Sapporo	LC199284	LC199287	—	LC199290	LC199295
<i>Ganaspis brasiliensis</i> unknown host (12)	Tokyo	LC199250	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (17)	Tokyo	LC199254	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (19)	Tokyo	LC199255	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (20)	Tokyo	LC199256	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (23)	Tokyo	LC199259	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (27)	Tokyo	LC199280	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (28)	Tokyo	LC199265	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (29)	Tokyo	LC199266	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (30)	Tokyo	LC199267	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (36)	Tokyo	LC199273	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (40)	Tokyo	LC199281	—	—	—	—
<i>Ganaspis brasiliensis</i> unknown host (42)	Tokyo	LC199278	—	—	—	—

Sample names consist of species name, host species, and individual number. Accession numbers for fragments obtained from NCBI database are shown in the tree tip labels in Figs. 2 and 3. Fragments that were determined but not used in the phylogenetic analysis are shown in parentheses.

Table 2 Proportion of female offspring in cross experiments using the KS and SP strains and the TK strain of *Ganaspis brasiliensis*. In crosses between females and males of the same strains, only the production of F₁ offspring was examined

	Female	Male		
		Group 5		Group 3
		KS	SP	TK
F1	KS	0.35 (536)	0.4 (1206)	0.0 (1433)
	SP	0.20 (1288)	0.37 (421)	0.002 (1276)
	TK	0.13 (99)	0.29 (107)	0.28 (299)
F2	KS	-	0.42 (2527)	-
	SP	0.44 (1810)	-	0.67 (57)
	TK	0.14 (358)	0.28 (603)	-
F3	KS	-	0.55 (1835)	-
	SP	0.60 (582)	-	0.41 (524)
	TK	0.54 (701)	0.53 (1574)	-

Figures in parenthesis refer to the total number of offspring obtained.

The KS and SP strains belong to group 5 of the phylogenetic trees based on *CO1* and the TK strain to group 3 (see Fig. 2).

Figure legends

Fig. 1 Collection localities

Fig. 2 Bayesian phylogenetic trees for *CO1*. The tree represents the maximum clade credibility tree with mean tree heights. Only posterior probabilities above 0.5 are displayed on the nodes. Accession numbers were given to the sequences obtained from the NCBI database. Abbreviations indicate host species and localities where the specimens originated; Deug (*D. eugracilis*), Dlut (*D. lutescens*), unk (unknown), Dtak (*Drosophila* sp. affi. *takahashii*), Dfic (*D. ficusphila*), Dsuz (*D. suzukii*), Ddar (*D. daruma*), Dalb (*D. albomicans*), Scor (*Scaptodrosophila coracina*), Dbiz (*D. bizonata*), BG (Bogor), CB (Cibodas), HW (Hawaii), IR (Iriomote-jima), KB (Kinabalu), KG (Kagoshima), KS (Kaohsiung), SD (Sendai), TK (Tokyo), UG (Uganda). G1–G5 indicate groups 1–5 (see text).

Fig. 3 Bayesian phylogeny tree for nDNA (ITS1, ITS2, *RpL37*). The tree represents the maximum clade credibility tree with mean tree heights. Only posterior probabilities above 0.5 are displayed on the nodes. Accession numbers were given to the sequences obtained from the NCBI database. Abbreviations indicate host species and localities where the specimens originated; TL (Thailand), PP (Philippines). For other abbreviations, see the legend of Fig. 2. G1–G5 indicate groups 1–5 recognized by the phylogenetic analysis with *CO1* (see text).

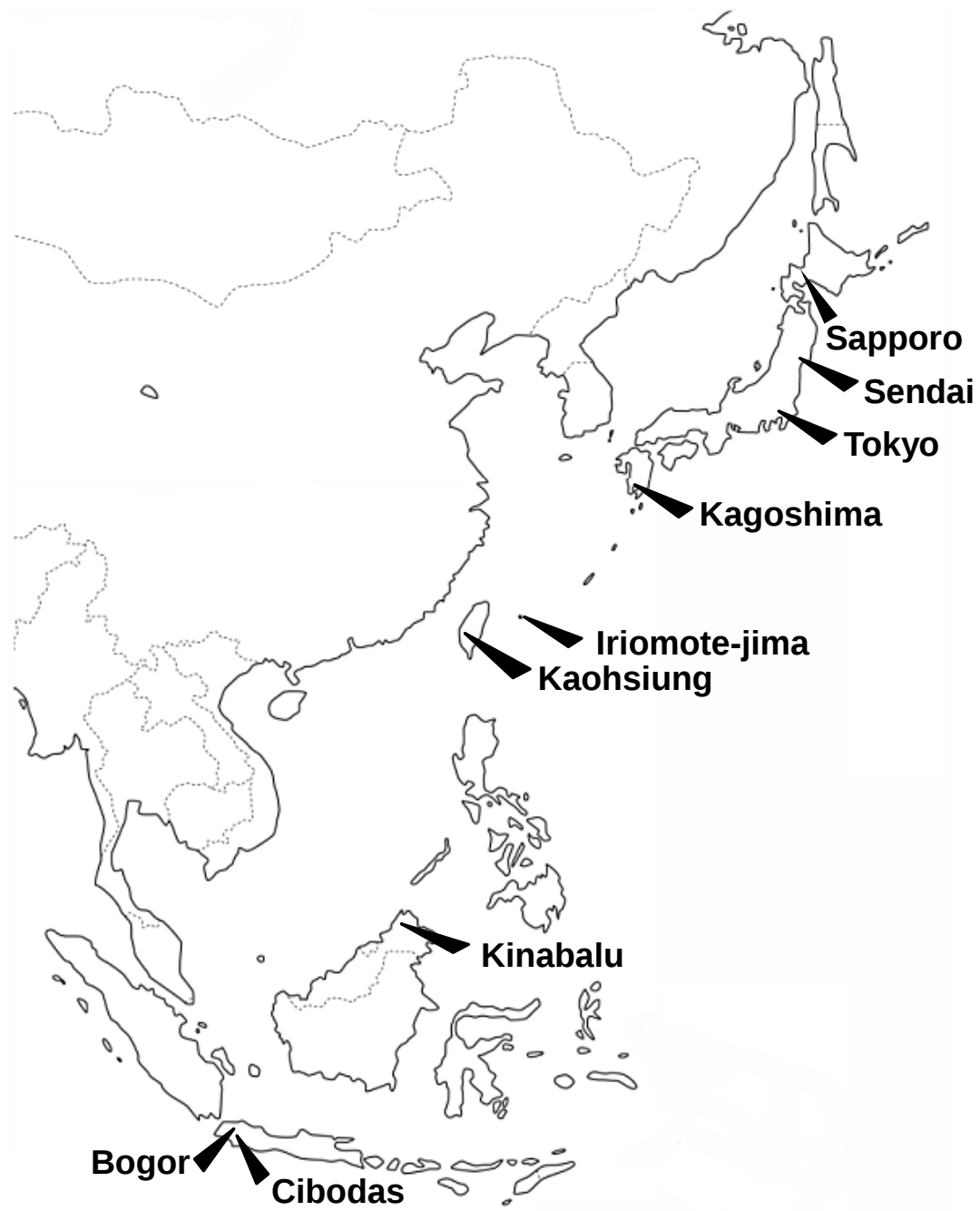


Fig. 1

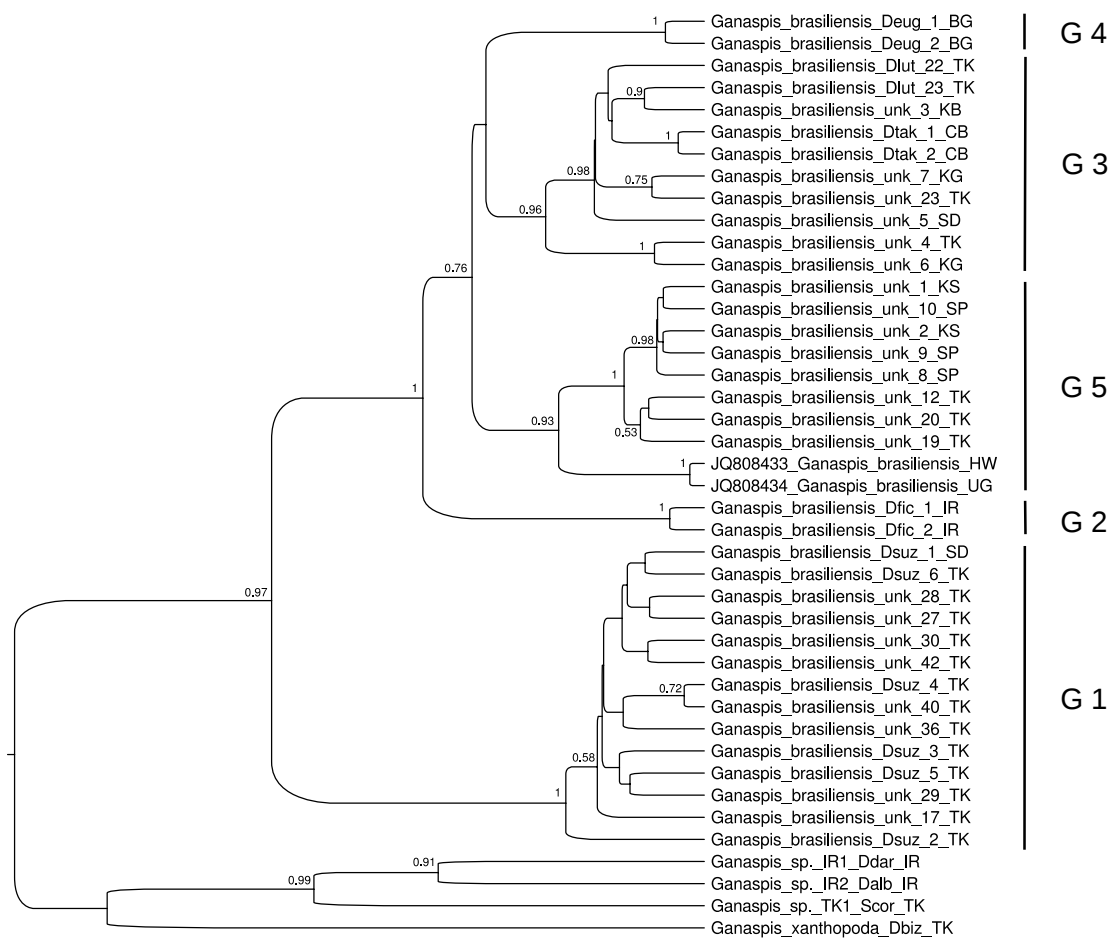


Fig. 2

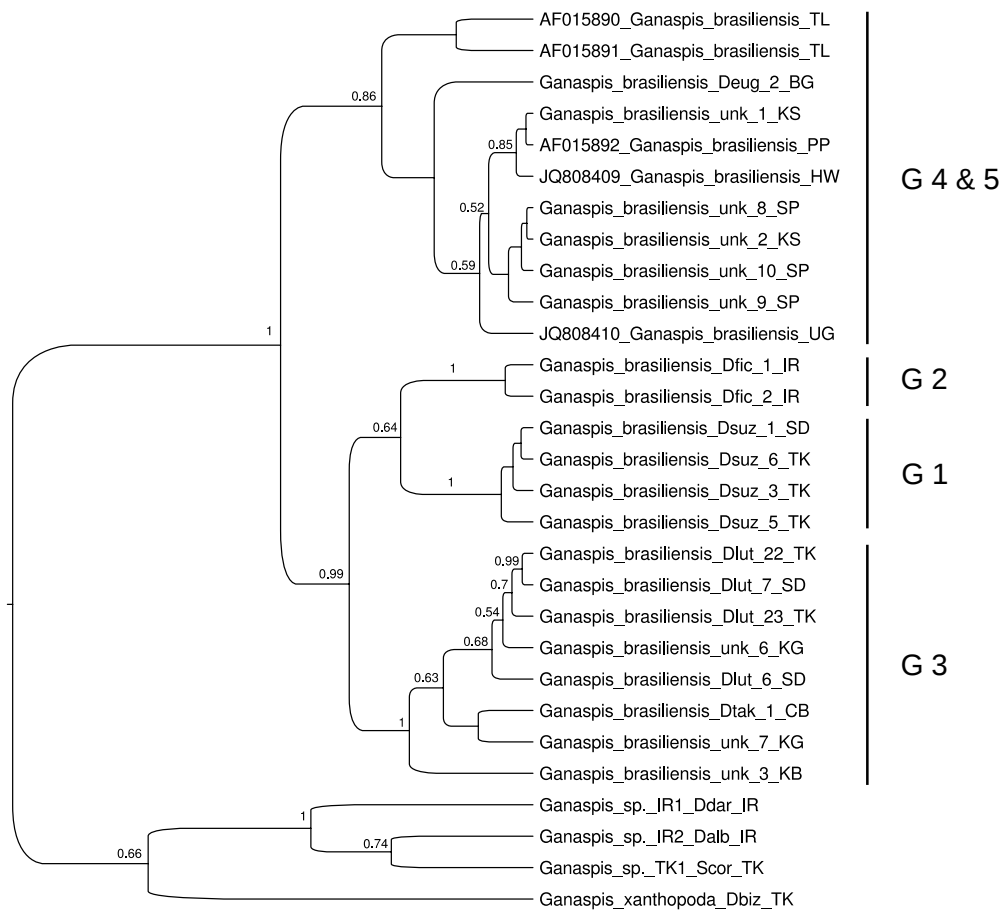


Fig. 3