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

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31

32 **Abstract**

33

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

49

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

52 **Introduction**

53

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

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

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

93

94 **Materials and methods**

95

96 Samples

97

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

108

109 Molecular methods

110

111 DNA was extracted from samples using DNeasy Blood & Tissue Kit (Qiagen, Hilden,
112 Germany). Target fragments were amplified by polymerase chain reaction (PCR). For
113 *COI*, two separate regions were amplified with the following primer pairs, LCO/HCO
114 (LCO: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO:
115 5'-TAAACTTCAGGGTGACCAAAAATCA-3', 440–688 bp; Folmer et al. 1994) and
116 hco-extA/hco-extB (hco-extA: 5'- GAAGTTTATATTTTAATTTTACCTGG-3' and
117 hco-extB: 5'-CCTATTGAWARAACATARTGAAAATG-3', 326–376 bp; Schulmeister

118 et al. 2002). ITS1, ITS2, and *RpL37* fragments were amplified using primer pairs,
119 7246/7247, I-2a/I-2b, and 27F/27R (Lohse et al. 2010), respectively (7246:
120 5'-GCTGCGTTCTTCATCGAC-3' and 7247: 5'-CGTAACAAGGTTTCCGTAGG-3',
121 241–736 bp; I-2a: 5'-TGTCAACTGCAGGACACATG-3' and I-2a:
122 5'-AATGCTTAAATTTAGGGGGTA-3', 239–531 bp; 27F:
123 5'-GAARGGTACNTCVAGYTTTGG-3', 27R:
124 5'-GACCRGTDCCRGTRGTCTTCCT-3', 520–766 bp). For samples that did not
125 amplify with 27F/27R, a reverse primer, 7g2r
126 (TGCTWATTTCTACTTATTTCAATTGCT), was developed using Primer3
127 (Untergasser et al. 2012) and paired with 27F. The reaction was performed in a mixture
128 containing 1.0 µl sample DNA, 2.0 µl 10× buffer, 2.5 mM MgCl₂, 100 µM dNTP, 0.5
129 µM each primer and 0.5 U AmpliTaq DNA polymerase (Applied Biosystems) in total
130 volume of 20 µl. The thermal profile for *COI*, ITS1 and ITS2 consisted of 94 °C for 10
131 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min, followed by
132 final extension at 72 °C for 1.5 min. *RpL37* was amplified with a touch down PCR
133 consisting of 94 °C for 3 min, 10 cycles of 94 °C for 15 s, 60–50 °C for 40 s, and 72 °C
134 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
135 by 72 °C for 10 min. Prism BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied
136 Biosystems) was used for sequence reactions. Sequencing was conducted with an ABI
137 3100 automated sequencer.
138
139 Phylogenetic analysis

140

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).

157

158 Crossing experiments

159

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

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

182

183 **Results**

184

185 Phylogenetic analysis

186

187 Figure 2 shows a tree based on the nucleotide sequences of the *COI* region, and Fig. 3

188 shows a tree based on concatenated ITS1, ITS2 and *RpL37* sequences. Both trees

189 revealed that *G. brasiliensis* and *G. xanthopoda* were distantly related among *Ganaspis*

190 species studied here. In the *COI* tree, individuals morphologically identified as *G.*

191 *brasiliensis* can be subdivided into five groups, 1) individuals parasitizing *D. suzukii*, 2)

192 those from Iriomote-jima (IR), 3) those from temperate lowlands of Japan (TK, SD:

193 Sendai, KG: Kagoshima) and high-altitude areas of tropical regions (CB: Cibodas, KB:

194 Mt. Kinabalu), 4) those from Indonesia (BG: Bogor), and 5) those from Japan (TK:

195 Tokyo and SP: Sapporo), Taiwan (KS: Kaohsiung), Uganda (UG) and Hawaii (HW).

196 The last group can be further subdivided into two subgroups; i.e., those from Japan (TK

197 and SP) and Taiwan (KS) and those from UG and HW. The nDNA tree agrees well with

198 the *COI* tree, except that the groups 4 and 5 in the *COI* tree are not clearly

199 discriminated. Individuals from the Philippines and Thailand are included in a complex

200 of the groups 4 and 5.

201 For the *COI* sequences, genetic distances between individuals of the 5 groups

202 were calculated with Kimura's two-parameter model and pairwise deletion. The genetic

203 distances between individuals of group 1 and those of groups 2, 3, 4 and 5 ranged from

204 0.047 to 0.071, and the distances between individuals of group 2 and those of groups 3,

205 4 and 5 ranged from 0.031 to 0.043. On the other hand, the distances between

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

207

208 Crossing experiments

209

210 Table 2 shows the results of cross experiments. The sex ratio was significantly deviated
211 from 1:1 in all cases where progenies were obtained (χ^2 test with sequential Bonferroni
212 correction, $p < 0.05$). In the crosses between females and males from the same strains,
213 the sex ratio F₁ offspring was male biased. In the cross between KS and SP, the sex ratio
214 of F₁ offspring was also male biased, but the sex ratio of F₂ and F₃ offspring was closer
215 to 1:1. In the cross between KS or SP females and TK males, almost only male
216 offspring was obtained, probably because mating did not occur. In the cross between SP
217 females and TK males, F₂ and F₃ offspring were obtained, and their sex ratio was closer
218 to 1:1 than F₁ offspring. In the cross between TK females and KS or SP males, F₁
219 offspring were produced, but their number was not large in comparison with other
220 crosses. In these crosses, the sex ratio was male biased in F₁ and F₂ offspring, but closer
221 to 1:1 in F₃ offspring.

222

223 **Discussion**

224

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

228 (1998) are revealed as *G. brasiliensis* as well as those reported by Mitsui et al. (2007),
229 Mitsui and Kimura (2010), Kasuya et al. (2013b) and Kimura and Suwito (2012, 2015).
230 Other *Ganaspis* individuals so far assigned as *G. xanthopoda* by *Drosophila* researchers
231 (e.g., Carton et al. 1986) would also be *G. brasiliensis*.

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

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

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

250 The third lineage is a generalist; it mainly parasitizes *Drosophila lutescens*, *D.*
251 *rufa* Kikkawa & Peng and *D. bauraria* Bock & Wheeler in Japan (Mitsui and Kimura
252 2010) and previously assigned as the *lutescens*-associated type of *G. xanthopoda* by
253 Kasuya et al. (2013b). Females of this lineage do not oviposit in *D. suzukii* larvae
254 (Mitsui and Kimura 2010). The geographic distribution of this lineage is unique; it
255 occurs in tropical highlands and temperate lowlands (Kimura and Suwito 2015; Mitsui
256 and Kimura 2010). Interestingly, a similar pattern of distributions is known for its host
257 *Drosophila* species, although its hosts in temperate lowlands and tropical highlands are
258 not conspecific; i.e., *D. lutescens* is distributed in temperate lowlands of Asia whereas
259 its close relatives such as *Drosophila* sp. aff. *takahashii* and *D. trilineata* are distributed in
260 tropical and subtropical highlands, and *D. rufa* and *D. bauraria* occur in temperate
261 lowlands whereas their relative *D. trapezifrons* Okada occurs in subtropical highlands
262 (Goto et al. 2000; Kimura and Suwito 2015; Kimura et al. 1994). This suggests a
263 possibility that this lineage of *G. brasiliensis* has expanded the distribution
264 corresponding to the distributions of host species.

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

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

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

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

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

296 It is noteworthy that the three lineages coexisted at least in Tokyo. If
297 genetically differentiated populations are present sympatrically, they are generally
298 recognized as different species. However, reproductive isolation between lineages 3 and
299 4 is incomplete, and the genetic distance between them was not high (0.013–0.025). As
300 mentioned before, lineage 4 may be an invasive species and may have recently
301 colonized Japan. If this is the case, it is worth investigating whether these two lineages
302 fuse upon hybridization or continue differentiation sympatrically. On the other hand, the
303 *suzukii*-associated type (lineage 1) differed 4–5 % from the other lineages in the *COI*
304 sequences, suggesting a possibility that it has differentiated from the others at species
305 level. To determine the species status of this type, it is needed to conduct mating
306 experiments.

307

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315

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

Samples	Locality	COI LCO/HCO	COI 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 *COI* and the TK strain to group 3 (see Fig. 2).

Figure legends

Fig. 1 Collection localities

Fig. 2 Bayesian phylogenetic trees for *COI*. 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 *COI* (see text).



Fig. 1

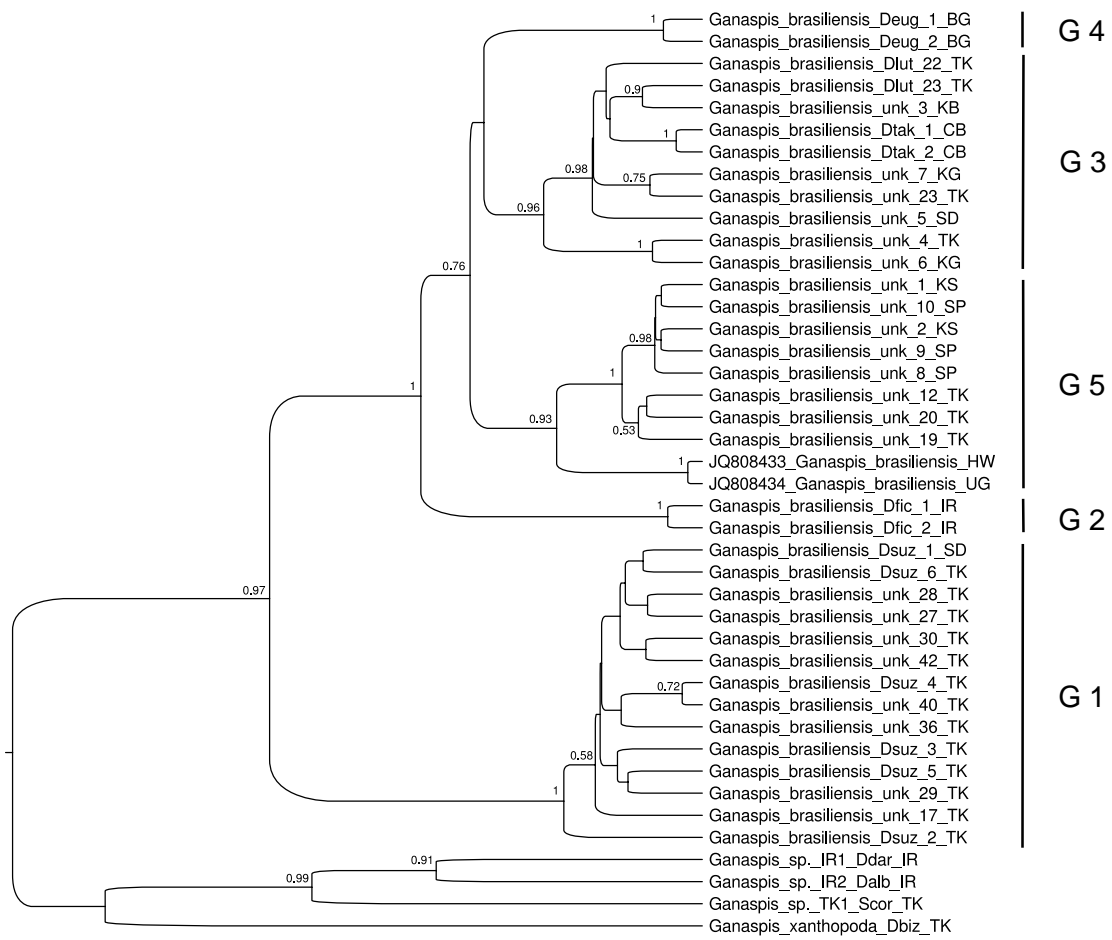


Fig. 2

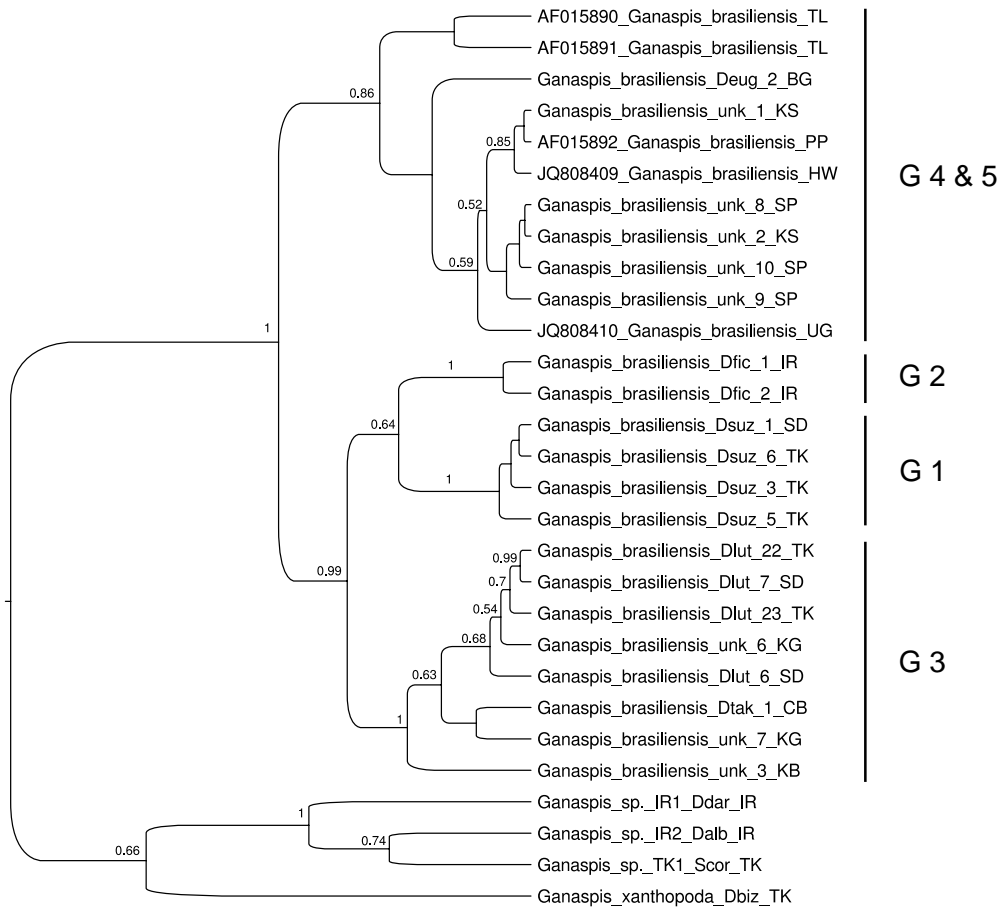


Fig. 3