Article type: Original research paper

Genetic differentiation of *Ganaspis brasiliensis* (Hymenoptera: Figitidae) from East 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. basiliensis* 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

96 Samples

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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.

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109 Molecular methods

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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 *CO1*, two separate regions were amplified with the following primer pairs, LCO/HCO
114 (LCO: 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO:
115 5′-TAAACTTCAGGGTGACCAAAAAATCA-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

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 $MgCl_2$, 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

All the sequences were aligned and adjusted by eye in MEGA 5.2 (Tamura et al. 2011). Substitution models were chosen with BIC (Bayesian information criterion) calculated by jModelTest (Darriba et al. 2012). The model for *CO1* sequences was GTR + Γ. ITS1, ITS2, and *RpL37* fragments were concatenated to estimate a single nDNA tree, with separate substitution models (HKY+Γ, HKY+I, and HKY+Γ, respectively). Rate variation across branches was assumed to follow exponential distribution (relaxed-clock model with uncorrelated rates; Drummond et al. 2006), and it was validated against strict-clock (Bayes factor for *CO1* = 34.23 ; nDNA = 22.01) (Baele and Lemey 2013; Kass and Raftery 1995). Models were fitted using BEAST2 (Bouckaert et al. 2014) and the convergence was confirmed in Tracer (Rambaut et al. 2014). Trees were visualized using FigTree (Ranbaut 2014). The sequences of *G. brasiliensis* (assigned as *G. xanthopoda*) from other locations (Hawaii, Philippines, Thailand, and Uganda) were obtained from NCBI database and used for the reconstruction of phylogenetic trees.

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

Crossing experiments

The level of reproductive isolation among the KS, TK and SP strains was examined by 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 F_1 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 F_1 males and females emerged, two to five F_1 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 F_2 and F_3 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

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185 Phylogenetic analysis

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187 Figure 2 shows a tree based on the nucleotide sequences of the *CO1* 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 *CO1* 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 *CO1* tree, except that the groups 4 and 5 in the *CO1* 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 *CO1* 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.

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208 Crossing experiments

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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 F_1 offspring was male biased. In the cross between KS and SP, the sex ratio
214 of F_1 offspring was also male biased, but the sex ratio of F_2 and F_3 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, F_2 and F_3 offspring were obtained, and their sex ratio was closer
218 to 1:1 than F_1 offspring. In the cross between TK females and KS or SP males, F_1
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 F_1 and F_2 offspring, but closer
221 to 1:1 in F_3 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 *CO1* 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.

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. trilutea* 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

272 in this lineage in the *CO1* 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 F_2
284 and F_3 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 *CO1*, 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 *CO1*
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
308 **Acknowledgments** We thank M. Kondo and M. B. Lankim who provided us *Ganaspis*
309 specimens from Kinabalu and T. I. Kohyama, G. Kinoshita, H. Suzuki for their advice
310 and assistance on the molecular and phylogenetic analyses. This study was supported by
311 Grant-in-Aids from the Ministry of Education, Science, Sports, Culture and Technology
312 of Japan (No. 23370005) and also performed as a part of the Dropsa project. The USDA
313 does not endorse any commercial product mentioned in this research. USDA is an equal
314 opportunity provider and employer.
315

316 **References**

317

318 Ashmead WH (1896) Report on the parasitic Hymenoptera of the island of Grenada,
319 comprising the families Cynipidae, Ichneumonidae, Braconidae, and
320 Proctotrypidae. Proc Zool Soc London 63:742–812
321 Asplen MK, Anfora G, Biondi A et al (2015) Invasion biology of spotted wing
322 Drosophila (*Drosophila suzukii*): a global perspective and future priorities. J
323 Pest Sci 88:469–495
324 Baele G, Lemey P (2013) Bayesian evolutionary model testing in the phylogenomics
325 era: matching model complexity with computational efficiency. Bioinformatics
326 29:1970–1979
327 Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu C-H, Xie D, Suchard MA, Rambaut
328 A, Drummond AJ (2014) BEAST 2: a software platform for Bayesian
329 evolutionary analysis. PLoS Comput Biol 10:e1003537.
330 Bruck, DJ, Bolda M, Tanigoshi L, Klick J, Kleiber J, DeFrancesco J, Gerdeman B,
331 Spitler H (2011) Laboratory and field comparisons of insecticides to reduce
332 infestation of *Drosophila suzukii* in berry crops. Pest Manag Sci 67:1375–1385
333 Buffington ML, Forshage M (2016) Redescription of *Ganaspis brasiliensis* (Ihering,
334 1905), new combination, (Hymenoptera: Figitidae) a natural enemy of the
335 invasive *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae). Proc
336 Entomol Soc Wash 118:1–13
337 Carton Y, Boulétreau M, van Alphen JJM, van Lenteren JC (1986) The *Drosophila*

parasitic wasps. In: Ashburner M, Carson HL, Thompson JN (eds) The genetic and biology of *Drosophila*, 3e. Academic Press, New York, pp 347–394

Daane KM, Wang X-G, Biondi A et al (2016) First exploration of parasitoids of *Drosoophila suzukii* in South Korea as potential classical biological agents. J Pest Sci 89:823–835

Darriba D, Taboada, GL, Doallo R, Posada D (2012). jModelTest 2: more models, new heuristics and parallel computing. Nat methods, 9:772–772.

Dobzhanski T (1965) “Wild” and “Domestic” species of Drosophila. In: Baker HG, Stebbins GL (eds) The genetics of colonizing species. Academic Press, New York, pp 533–546

Drummond AJ, Ho S, Phillips M, Rambaut A (2006) Relaxed phylogenetics and dating with confidence. PLoS Biol 4:e88

Folmer O, Black M, Hoeh W, Luiz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294–299

Goto SG, Kitamura HW, Kimura MT (2000) Phylogenetic relationships and climatic adaptations in the *Drosophila takahashii* and *montium* species subgroups. Mol Phyl Evol 15:147–156

Kacsoh BZ, Schlenke TA (2012) High hemocyte load is associated with increased resistance against parasitoids in *Drosophila suzukii*, a relative of *D. melanogaster*. PLoS ONE 7:e34721

Kanzawa T (1939) Studies on *Drosophila suzukii* Mats. Yamanashi Agri Exp Sta Rep

Kofu, Japan (In Japanese)

Kass RE, Raftery AE (1995) Bayes factors. J Am Stat Assoc 90:773–795

Kasuya N, Mitsui Y, Aotsuka T, Kimura M T (2013a) Diversity and host association of parasitoids attacking mycophagous drosophilids (Diptera: Drosophilidae) in northern and central Japan. Entomol Sci 16:227–234

Kasuya N, Mitsui H, Ideo S, Watada M, Kimura MT (2013b) Ecological, morphological and molecular studies on *Ganaspis* individuals (Hymenoptera: Figitidae) attacking *Drosophila suzukii* (Diptera: Drosophilidae). Appl Entomol Zool 48:87–92

Kimura MT (2015) Prevalence of exotic frugivorous *Drosophila* species, *D. simulans* and *D. immigrans* (Diptera: Drosophilidae), and its effects on local parasitoids in Sapporo, northern Japan. Appl Entomol Zool 50:509–515

Kimura MT, Suwito A (2012) Diversity and abundance of frugivorous drosophilids and their parasitoids in Bogor, Indonesia. J Nat Hist 46:1947–1957

Kimura MT, Suwito A (2015) Altitudinal patterns of abundances and parasitism in frugivorous drosophilids in west Java, Indonesia. J Nat Hist 49:1627–1639

Kimura MT, Ohtsu T, Yoshida T, Awasaki T., Lin FJ (1994) Climatic adaptation and distributions in the *Drosophila takahashii* species subgroup (Diptera: Drosophilidae). J Nat Hist 28:401–409

Lemeunier F, Tsacas L, David J, Ashburner M (1986) The *melanogaster* species group. In: Thompson JR, Carson HL (eds) The genetics and biology of *Drosophila*, 3e. Academic press, New York, pp 147–256

Lohse K, Sharanowski B, Stone GN (2010) Quantifying the pleistocene history of the oak gall parasitoid *Cecidostiba fungosa* using twenty intron loci. Evolution 64:2664–2681

Mitsui H, Kimura MT (2010) Distribution, abundance and host association of two parasitoid species attacking frugivorous drosophilid larvae in central Japan. Eur J Entomol 107:535–540

Mitsui H, Van Achterberg K, Nordlander G, Kimura MT (2007) Geographical distributions and host associations of larval parasitoids of frugivorous Drosophilidae in Japan. J Nat Hist 41:1731–1738

Paradis E, Claude J, Strimmer K (2004) APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20:289–290.

R Core Team (2015) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.r-project.org/. Accessed 17 August 2015

Ranbaut A (2014). FigTree ver. 1.4.2. http://tree.bio.ed.ac.uk/software/figtree. Accessed 13 February 2015

Rambaut A, Suchard MA, Xie D, Drummond A (2014) Tracer ver. 1.6. http://beast.bio.ed.ac.uk/Tracer. Accessed 1 May 2015

Schilthuizen M, Nordlander G, Stouthamer R, van Alphen JJM (1998) Morphological and molecular phylogenetics in the genus *Leptopilina* (Hymenoptera: Cynipoidea: Eucoilidae). Syst Entomol 23:253–264

Schulmeister S, Wheeler WC, & Carpenter JM (2002) Simultaneous analysis of the

404 basal lineages of Hymenoptera (Insecta) using sensitivity analysis. Cladistics
405 18:455–484
406 Tamura K, Petersen D, Petersen N, Stecher G, Nei M, Kumar S (2011) MEGA5:
407 molecular evolutionary genetics analysis (MEGA) using maximum likelihood,
408 evolutionary distance, and maximum parsimony methods. Mol Biol Evol
409 28:2731–2739
410 Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG
411 (2012) Primer3 - new capabilities and interfaces. Nucl Acid Res 40:e115

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

Ganaspis brasiliensis | *unknown host* (1)	Kaohsiung	LC122443	LC122042	LC120766	LC122346	–
Ganaspis brasiliensis | *unknown host* (2)	Kaohsiung	LC122455	LC122043	LC120762	LC122336	LC122576
Ganaspis brasiliensis | *unknown host* (3)	Mt. Kinabalu	LC122449	LC122044	LC120765	LC122342	LC122577
Ganaspis brasiliensis | *unknown host* (4)	Tokyo	AB456710	–	–	–	–
Ganaspis brasiliensis | *unknown host* (5)	Sendai	AB456711	–	–	–	–
Ganaspis brasiliensis | *unknown host* (6)	Kagoshima	LC122456	LC122052	LC120771	LC122347	LC122583
Ganaspis brasiliensis | *unknown host* (7)	Kagoshima	LC122457	LC122053	LC120772	LC122348	LC122582
Ganaspis brasiliensis | *unknown host* (8)	Sapporo	LC199282	LC199285	LC199291	LC199288	LC199293
Ganaspis brasiliensis | *unknown host* (9)	Sapporo	LC199283	LC199286	LC199292	LC199289	LC199294
Ganaspis brasiliensis | *unknown host* (10)	Sapporo	LC199284	LC199287	–	LC199290	LC199295
Ganaspis brasiliensis | *unknown host* (12)	Tokyo	LC199250	–	–	–	–
Ganaspis brasiliensis | *unknown host* (17)	Tokyo	LC199254	–	–	–	–
Ganaspis brasiliensis | *unknown host* (19)	Tokyo	LC199255	–	–	–	–
Ganaspis brasiliensis | *unknown host* (20)	Tokyo	LC199256	–	–	–	–
Ganaspis brasiliensis | *unknown host* (23)	Tokyo	LC199259	–	–	–	–
Ganaspis brasiliensis | *unknown host* (27)	Tokyo	LC199280	–	–	–	–
Ganaspis brasiliensis | *unknown host* (28)	Tokyo	LC199265	–	–	–	–
Ganaspis brasiliensis | *unknown host* (29)	Tokyo	LC199266	–	–	–	–
Ganaspis brasiliensis | *unknown host* (30)	Tokyo	LC199267	–	–	–	–
Ganaspis brasiliensis | *unknown host* (36)	Tokyo	LC199273	–	–	–	–
Ganaspis brasiliensis | *unknown host* (40)	Tokyo	LC199281	–	–	–	–
Ganaspis brasiliensis | *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 F_1 offspring was examined

		Male		
	Female	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

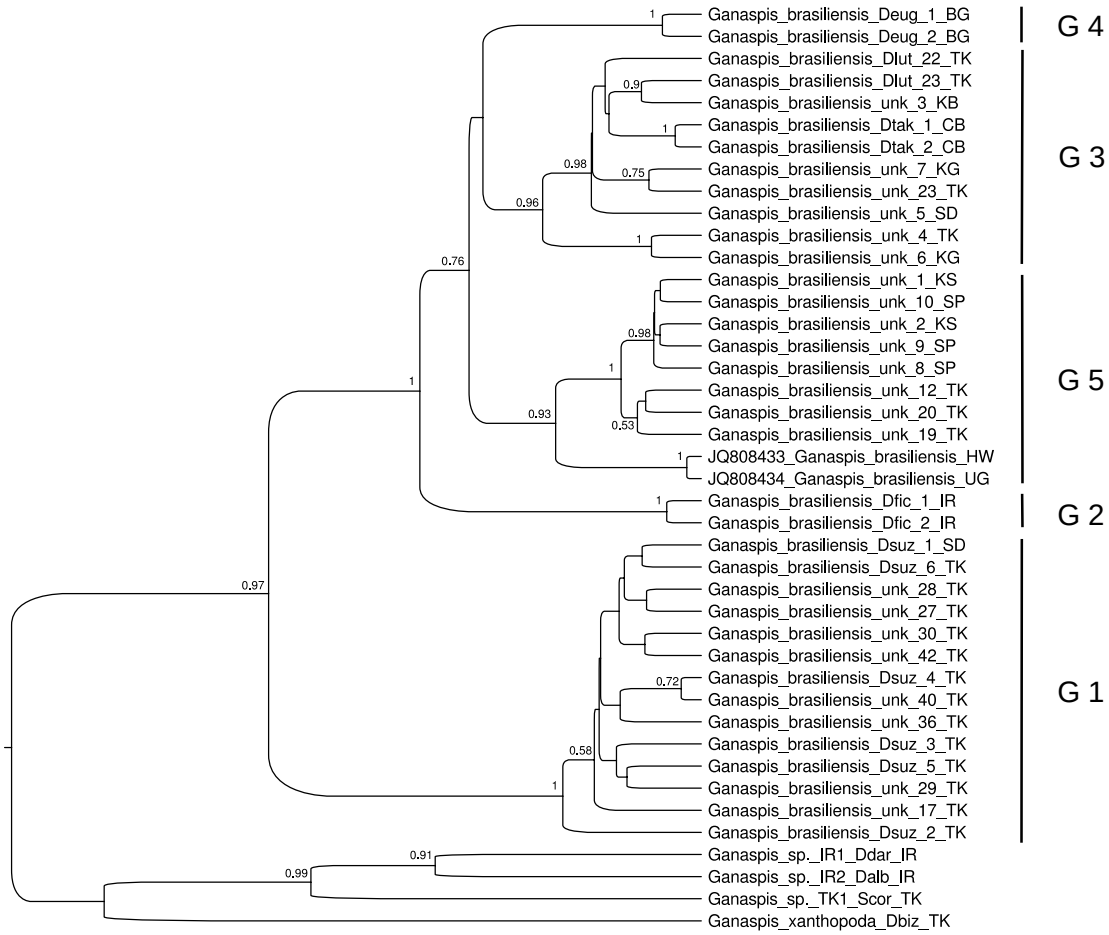
Fig. 1 Collection localities

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 (*Drosopohila* 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).


Sapporo
Sendai
Tokyo
Kagoshima
Iriomote-jima
Kaohsiung
Kinabalu
Bogor
Cibodas

Fig. 1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Ganaspis_brasiliensis_Deug_2_BG
Ganaspis_brasiliensis_Dlut_22_TK
Ganaspis_brasiliensis_Dlut_23_TK
Ganaspis_brasiliensis_unk_3_KB
Ganaspis_brasiliensis_Dtak_1_CB
Ganaspis_brasiliensis_Dtak_2_CB
Ganaspis_brasiliensis_unk_7_KG
Ganaspis_brasiliensis_unk_23_TK
Ganaspis_brasiliensis_unk_5_SD
Ganaspis_brasiliensis_unk_4_TK
Ganaspis_brasiliensis_unk_6_KG
Ganaspis_brasiliensis_unk_1_KS
Ganaspis_brasiliensis_unk_10_SP
Ganaspis_brasiliensis_unk_2_KS
Ganaspis_brasiliensis_unk_9_SP
Ganaspis_brasiliensis_unk_8_SP
Ganaspis_brasiliensis_unk_12_TK
Ganaspis_brasiliensis_unk_20_TK
Ganaspis_brasiliensis_unk_19_TK
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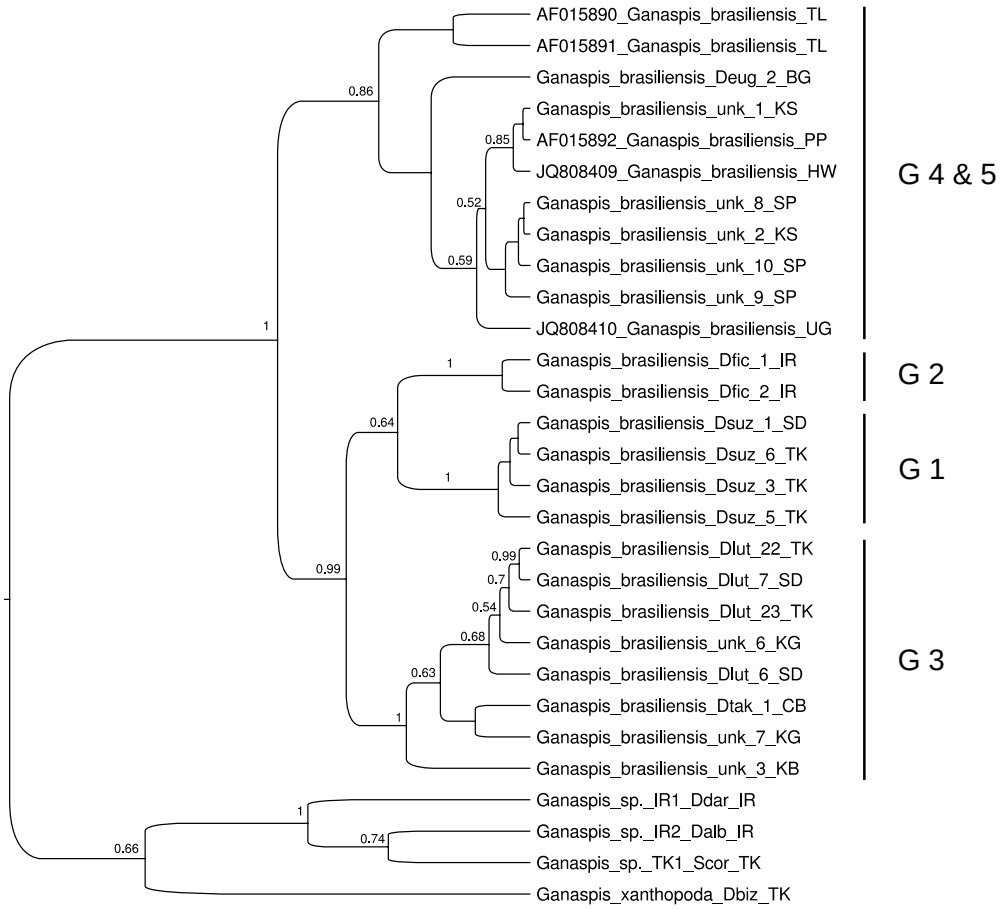
JQ808434_Ganaspis_brasiliensis_UG
Ganaspis_brasiliensis_Dfic_1_IR
Ganaspis_brasiliensis_Dfic_2_IR
Ganaspis_brasiliensis_Dsuz_1_SD
Ganaspis_brasiliensis_Dsuz_6_TK
Ganaspis_brasiliensis_unk_28_TK
Ganaspis_brasiliensis_unk_27_TK
Ganaspis_brasiliensis_unk_30_TK
Ganaspis_brasiliensis_unk_42_TK
Ganaspis_brasiliensis_Dsuz_4_TK
Ganaspis_brasiliensis_unk_40_TK
Ganaspis_brasiliensis_unk_36_TK
Ganaspis_brasiliensis_Dsuz_3_TK
Ganaspis_brasiliensis_Dsuz_5_TK
Ganaspis_brasiliensis_unk_29_TK
Ganaspis_brasiliensis_unk_17_TK
Ganaspis_brasiliensis_Dsuz_2_TK
Ganaspis_sp._IR1_Ddar_IR
Ganaspis_sp._IR2_Dalb_IR
Ganaspis_sp._TK1_Scor_TK
Ganaspis_xanthopoda_Dbiz_TK
G 4
G 3
G 5
G 2
G 1

Fig. 2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Ganaspis_brasiliensis_unk_8_SP
Ganaspis_brasiliensis_unk_2_KS
Ganaspis_brasiliensis_unk_10_SP
Ganaspis_brasiliensis_unk_9_SP
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Ganaspis_brasiliensis_Dfic_1_IR
Ganaspis_brasiliensis_Dfic_2_IR
Ganaspis_brasiliensis_Dsuz_1_SD
Ganaspis_brasiliensis_Dsuz_6_TK
Ganaspis_brasiliensis_Dsuz_3_TK
Ganaspis_brasiliensis_Dsuz_5_TK
Ganaspis_brasiliensis_Dlut_22_TK
Ganaspis_brasiliensis_Dlut_7_SD
Ganaspis_brasiliensis_Dlut_23_TK
Ganaspis_brasiliensis_unk_6_KG
Ganaspis_brasiliensis_Dlut_6_SD
Ganaspis_brasiliensis_Dtak_1_CB
Ganaspis_brasiliensis_unk_7_KG
Ganaspis_brasiliensis_unk_3_KB
Ganaspis_sp._IR1_Ddar_IR
Ganaspis_sp._IR2_Dalb_IR
Ganaspis_sp._TK1_Scor_TK
Ganaspis_xanthopoda_Dbiz_TK
0.86
0.85
0.52
0.59
1
1
0.64
1
0.99
0.99
0.7
0.54
0.68
0.63
1
1
0.74
0.66
G 4 & 5
G 2
G 1
G 3

Fig. 3