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First records of adventive populations of the parasitoids *Ganaspis brasiliensis* and *Leptopilina japonica* in the United States

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Abstract

We report the first known incidence of two parasitoid species of the invasive pest, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), in the United States (US). The discovery of *Ganaspis brasiliensis* (Ihering) (Hymenoptera: Figitidae) and *Leptopilina japonica* (Novković & Kimura) (Hymenoptera: Figitidae) in northwestern Washington State (US) was made shortly after their discovery in nearby southwestern British Columbia (Canada), indicating that contiguous populations of these species are established in both countries. The first specimen of *L. japonica* from Washington was collected in the fall of 2020, when it was found in a rice wine/orange juice trap deployed to survey for *Vespa mandarinia* Smith (Hymenoptera: Vespidae). Subsequent examination of trap contents from the 2020–2021 seasons indicated the presence of both *L. japonica* and *G. brasiliensis*. In September of 2021, live collections of both *G. brasiliensis* and *L. japonica* were made, reared from *D. suzukii*-infested Himalayan blackberry in Whatcom County, WA. Adult parasitoid identifications were based on morphology and COI DNA barcodes. All sequenced specimens to date from Washington and British Columbia belong to the G1 group of *G. brasiliensis*, the only group approved for release as a classical biological control agent in the US. This study provides an example of how even small changes in the geographic range of a natural enemy, now extending across an international border, may have significant consequences for the future of a biological control program.

Keywords
Adventive establishment, Drosophilidae, Figitidae, invasive species, spotted-wing drosophila

Introduction

The number of invasive insect pests being introduced to new geographic areas continues to increase (Seebens et al. 2017) and the subsequent discovery of introductions of their co-evolved natural enemies is also a common occurrence (Roy et al. 2011; Weber et al. 2021). "Unintentional biological control" occurs when natural enemies of invasive pests are unintentionally introduced to new areas; probably often *via* the same routes as their hosts, whose populations act as "receptive bridgeheads" (Weber et al. 2021). The phenomenon of unintentional biological control has begun in the Pacific Northwest (coastal areas of Oregon (OR), Washington (WA) in the United States (US) and British Columbia (BC), Canada) for a recent invasive pest, the spotted-wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae).

Drosophila suzukii was identified on the west coast of the US and Canada in 2008–2009, and its known distribution increased rapidly over the next few years (Asplen et al. 2015). Within a short period after the first detection, it was promoted to the status of a major, direct pest of cherries and berries, posing severe challenges to extant integrated pest management systems in these crops (Walsh et al. 2011). Early control efforts focused on insecticides (Beers et al. 2011; Bruck et al. 2011), which still form the core control tactic for *D. suzukii* despite a growing body of research on alternative controls (Tait et al. 2021). Research on biological controls found minimal contribution from natural enemies resident in the US (Miller et al. 2015; Lee et al. 2019; Tait et al. 2021). Generalist predators may have some impact in fields where fewer pesticides are applied (Lee et al. 2019), but both egg and early larval stages inside the fruit are inaccessible to predators. The most common parasitoids of *D. suzukii* in the Pacific Northwest are two pupal parasitoids, *Pachycrepoideus vindemmiae* (Rondani) (Hymenoptera: Pteromalidae) and *Trichopria drosophilae* (Perkins) (Hymenoptera: Diapriidae); however, parasitism occurs at low levels only, and was not thought to have a meaningful impact on *D. suzukii* populations (Miller 2015, Lee et al. 2019).

Because surveys of naturally occurring parasitoids attacking *D. suzukii* in North America and Europe showed low levels of parasitism, foreign explorations for co-evolved parasitoids were made in South Korea, China and Japan (Daane et al. 2016; Girod et al. 2018a; Giorgini et al. 2019). Several parasitoids attacking *D. suzukii* were reported, with *Ganaspis brasiliensis* (Ihering) and *Leptopilina japonica* (Novković & Kimura) (Hymenoptera: Figitidae) as the dominant parasitoids. To determine the suitability of these parasitoids for classical biological control, studies of their biology and host range were conducted in quarantine facilities in Switzerland (Girod et al. 2018b, c) and California (Wang et al. 2018; Hougardy et al. 2019; Wang et al. 2019, 2020; Biondi et al. 2021). Some populations of *G. brasiliensis* appear to be more specialized on *D. suzukii*, whereas other populations have broader host ranges (Girod et al. 2018b; Seehausen et al. 2020; Daane et al. 2021). Nomano et al. (2017) grouped *G. brasiliensis* into five "lineages" (G1–G5), hereafter referred to as "groups", based on molecular analyses and suggested that G1 material collected in Japan was host specific to *D. suzukii*. Collections of *G. brasiliensis* reared from *D. suzukii* in Asia have been a mixture of G1 and G3 groups, with G1 as the dominant group reared from *D. suzukii* (Giorgini et al. 2019). Moreover, recent reports confirmed that G1 populations in Japan specialize on *D. suzukii* and Seehausen et al. (2020) suggest G1 and G3 are, in fact, cryptic species, with G3 having a slightly broader host range (Seehausen et al. 2020; Daane et al. 2021). Based on the quarantine studies in Switzerland and California, including the host specificity studies of the G1 group, a petition submitted to USDA APHIS was approved for the release of the G1 *G. brasiliensis* in the US.

Federal approval to release G1 *G. brasiliensis* in the US impacts the future use of any adventive parasitoid populations found in North America and *vice versa*. In fact, both *L. japonica* and *G. brasiliensis* were reported to be established in 2019 in the south coastal region of British Columbia, Canada, representing the first time that the *G. brasiliensis* had been reported in North America and the first time that *L. japonica* had been detected outside of Asia (Abram et al. 2020). Shortly afterwards, the first report of *L. japonica* in Europe (Italy) was published (Puppato et al. 2020). These adventive populations of larval parasitoids of *D. suzukii* on both continents are probably the result of unintentional introductions. Adventive populations of *G. brasiliensis* and *L. japonica* in British Columbia appear to be well established, co-existing and parasitizing *D. suzukii* larvae in diverse habitats on a wide variety of different host plants over the course of the growing season (Abram et al. 2022a). However, exotic parasitoids collected in Canada cannot be intentionally released into the US. For that reason, whether the two species' distributions extend across the Canada-US border is of great interest to biological control practitioners. In this study, we report the discovery of *G. brasiliensis* and *L. japonica* in the northwestern Washington State (US), found in close proximity to the populations found in adjacent southwestern British Columbia.

Materials and methods

Bait traps

The Washington State Department of Agriculture conducted a detection-eradication program for the giant hornet, *Vespa mandarinia* Smith (Hymenoptera: Vespidae) in 2020 and 2021 in northwestern Washington. The traps consisted of plastic bottles baited with 250 ml of a 50:50 mixture of rice wine and orange juice. The traps attracted large numbers of nontarget Diptera and Hymenoptera, including *D. suzukii* and hymenopterous parasitoids. Based on the report of parasitoids of *D. suzukii* in nearby British Columbia, the by-catch from several thousand traps collected in August through November 2020 was screened for species of Figitidae.

Fruit collection

In September of 2021, we sampled for parasitoids of *D. suzukii* near Lynden, WA (48.94°N, 122.45°W) (Whatcom County), ~1.37 km south of the Canadian border. The site was a strip of unmanaged vegetation bordering a small creek between fields of commercial blueberry (*Vaccinium corymbosum* (Linnaeus) (Ericales: Ericaceae)) and raspberry (*Rubus idaeus* (Linnaeus) (Rosales: Rosaceae)). The farm had a known history of *D. suzukii* infestation, and this pest was also presumed to occur in the wild Himalayan blackberry patches, *Rubus armeniacus* (Focke) (Rosales: Rosaceae), bordering the field. This was informally confirmed by observing *D. suzukii* adults on or near the fruits. We collected ~200 berries from a 300 m section of the field border and returned them to the Washington State University Tree Fruit Research and Extension Center in Wenatchee, WA. The fruit were placed in a single layer in a 40 × 32 × 15 cm plastic container lined with absorbent paper and placed in 45 × 45 × 45 cm insect cage (BugDorm, MegaView Science Co., Ltd., Taichung, Taiwan) and kept in a growth chamber at 23 °C 16L:8D. Between 13–15 October, 2021, approximately 100 parasitoid wasps emerged from the blackberries.

Female and male wasp specimens from the live collection were tentatively identified as belonging to the Eucolinae (Figitidae) using an identification key for parasitoids associated with *D. suzukii* (Abram et al. 2022b). Specimens were confirmed as *G. brasiliensis* (Fig. 1) and *L. japonica* (Fig. 2) by the USDA-ARS Systematic Entomology Laboratory in Washington, District of Columbia, US.

Comparisons of the Washington specimens were made with *L. japonica* and *G. brasiliensis* collected in the lower mainland region of coastal British Columbia (Abram et al. 2020), as well as specimens at the National Insect Collection, National Museum of Natural History, Smithsonian Institution. All vouchers of this study are retained therein.

The morphological diagnosis confirmed the two parasitoid species, and was supplemented by molecular diagnosis of one male and four females of *G. brasiliensis* and five females of *L. japonica*. The primary goal of sequencing *L. japonica* was to confirm species identification, whereas the main goal of sequencing *G. brasiliensis* (which morphologically is more distinct from the native parasitoid fauna) was to determine their group (G1–G5). For reared wasps, whole specimens were first photographed to record the key characters (Abram et al. 2022b), and then DNA was extracted from each wasp specimen's metasoma (preserving the head and mesosoma for diagnostic identification and vouchers) using a Qiagen DNeasy blood and tissue kit (Qiagen) following the manufacturer's protocol. For specimens collected in *V. mandarinia* traps, all legs were removed from the right side of the insect body and extracted in Chelex 100 resin in 30 µl volumes containing 10% Chelex, and 4% Protinase K (20 mg/mL) (for *G. brasiliensis*), or a Lucigen MasterPure kit (for *L. japonica*) following the manufacturer's protocol.

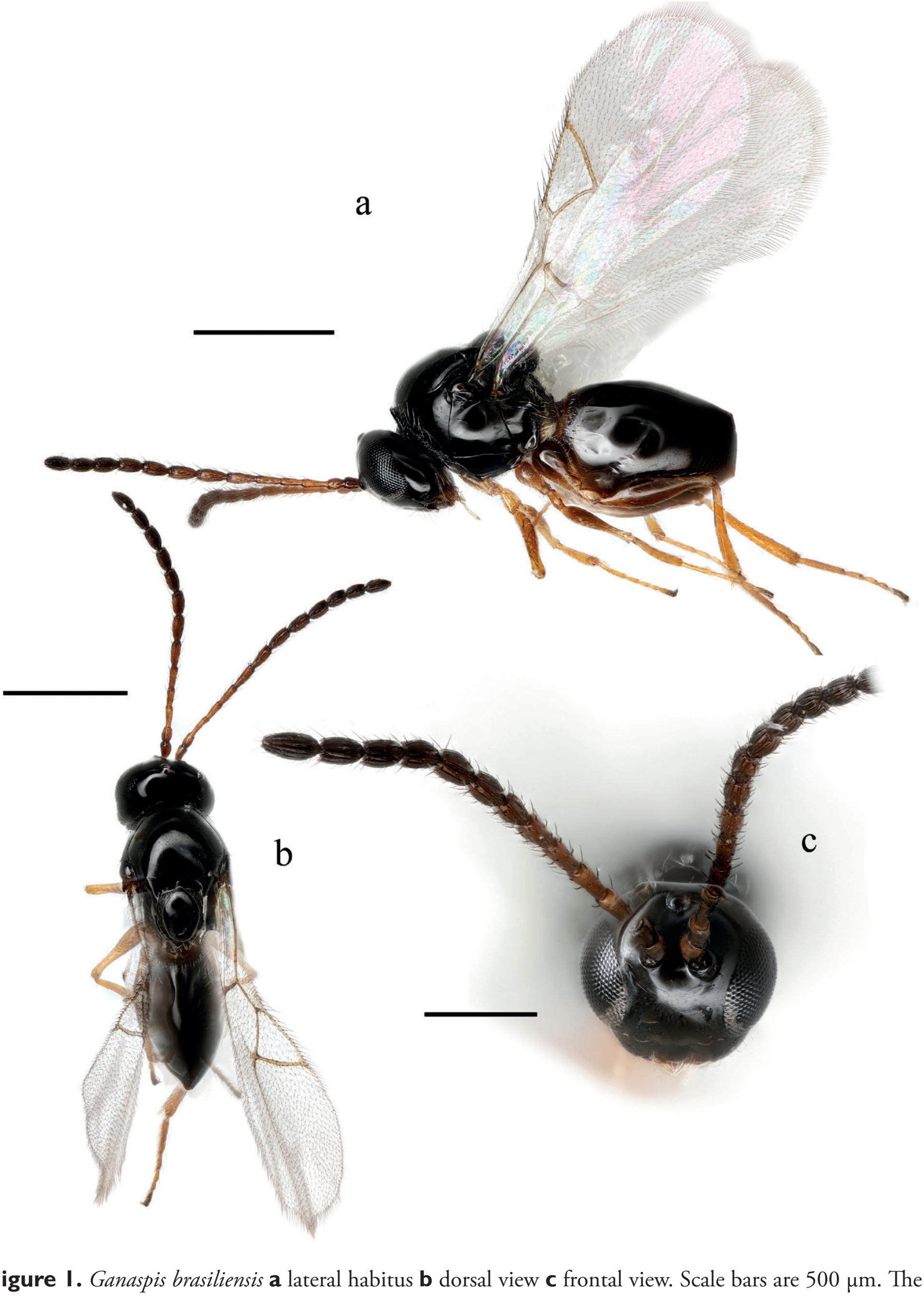

Figure 1. *Ganaspis brasiliensis* **a** lateral habitus **b** dorsal view **c** frontal view. Scale bars are 500 µm. The specimen was from the colony originally collected near Lynden, WA in 2021. Photo: Silas Bossert.

Previously described universal primers for the mitochondrial cytochrome oxidase subunit region (CO1), LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'), were used for molecular identification of parasitoids (Folmer et al. 1994). PCR reactions for reared specimens were performed using Advantage 2 Polymerase Mix (Takara Bio USA) in 25 µl reactions consisting of 0.5 µl Advantage 2 Polymerase Mix, 2.5 µl 10 X Advantage SA PCR Buffer, 0.2 mM dNTP, 0.1 µM primers, and 1 µl DNA. PCR conditions consisted of 94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 41 °C for 30 s, and 72 °C for 45 s; followed by 72 °C for 7 min. Reactions for specimens captured in *V. mandarinia* traps comprised 5 µl of molecular grade water, 12.5 µl of 2X Platinum II Hot-start Green PCR Master Mix (Invitrogen), 1.25 µl of $MgCl_2$ (50 mM), 0.5 µl of F and R primers (10 µM), and 2 µl of DNA template. LCO-1490 and HCO-2198 primers with M13 tails were used with cycling conditions as follows: 95 °C for 1 min., 35 cycles at 96 °C for 2 s, 50 °C for 5 s, and 72 °C for 20 s. Final extension was at 72 °C for 2 min. Amplicons produced under each sequencing approach were observed on 1.5% agarose gels stained with ethidium bromide. Remaining PCR products were cleaned with ExoSAP-IT™

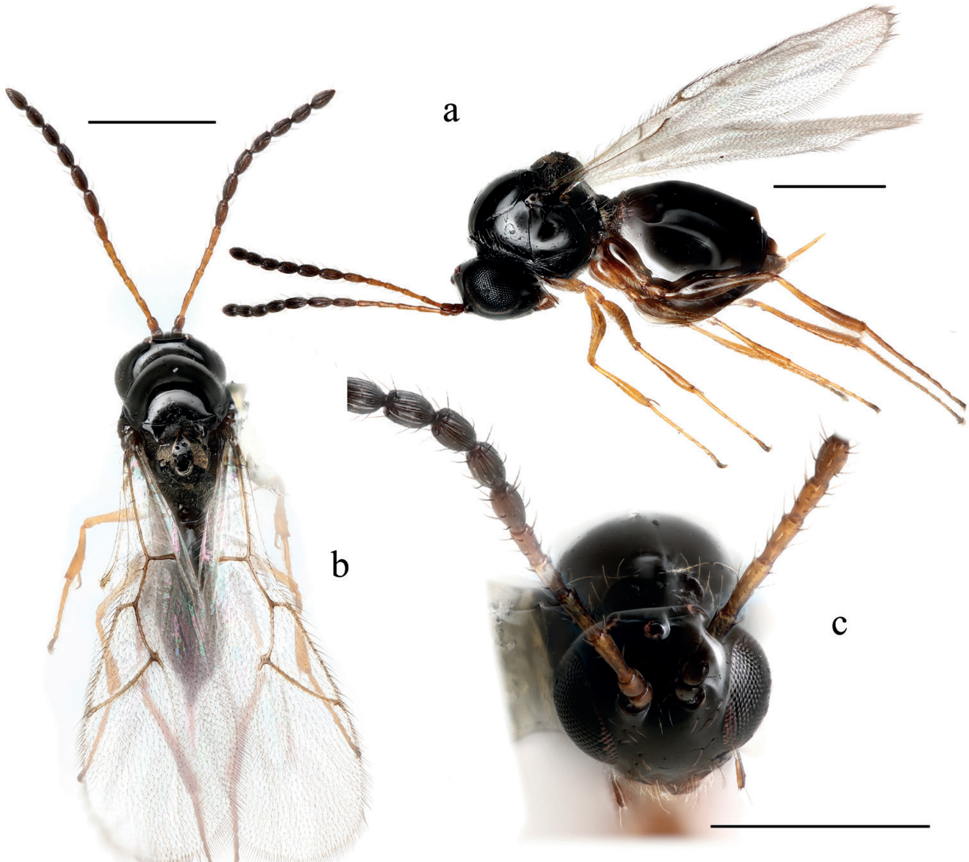

Figure 2. *Leptopilina japonica* **a** lateral habitus **b** dorsal view **c** frontal view. Scale bars are 500 µm. The specimen was from the colony originally collected near Lynden, WA in 2021. Photo: Silas Bossert.

(Applied Biosystems™, Thermo Fisher Scientific) and direct sequenced (Sanger method) (Mclab, San Francisco, CA).

For *G. brasiliensis*, the forward and reverse sequences of COI were reverse complemented into a final composite sequence using Sequencher 5.4.6. Three of five specimens (all females) yielded 96% complementary sequences, and these were then added to the *G. brasiliensis* master matrix of COI data. Alignment was trivial and done by eye; the resulting matrix was analyzed using Mr Bayes 3.2.2 for 1 mil generations, and trees visualized using TreeFig 1.4.2. One specimen of *L. japonica* generated a sequence that could be analyzed (92% complementary between forward and reverse). The COI sequence of *L. japonica* was added to the *Leptopilina* species sequence matrix that was selected from the DROP project (Lue et al. 2021), a molecular voucher database designed for parasitoids of *Drosophila* species in which sequences are linked to vouchers and vetted by taxonomists.

Five *G. brasiliensis* specimens from Abram et al. (2020) were previously sequenced, and four from the current study; eight of these were sampled from late season *R. armeniacus* from four different collection sites. The ninth sequence was obtained for a specimen collected in a *V. mandarinia* trap at an additional site in Washington, also collected late in the season. The small sample size leaves open the possibility that additional haplotypes could be present at other times of year on other host plants or locations. To test this possibility, we sequenced the COI region (using the same methods as described above) of an additional 36 *G. brasiliensis* specimens from collections made in 2020, details of which are reported in Abram et al. (2022a). These were from three different host plants and six different sites representing a variety of habitats from June to October (Table 1). Sequences could not be resolved from 3 of the specimens, resulting in conclusive haplotypes for 33 individuals.

Table 1. Haplotype designation of 37 specimens of *Ganaspis brasiliensis* from British Columbia (BC) and Washington (WA), 2020–2021.

Country (Province/ State), collection year	Site description	GPS	Plant host	Sampling date(s)	Number of specimens (sex)	*G. brasiliensis* COI haplotype
Canada (BC), 2020	Experimental farm	49.2435°N, 121.7552°W	*Rubus armeniacus*	Aug 26	1 (1 ♀)	G1
			Sambucus racemosa	Jul 8	3 (3 ♂)	G1
	Mid-elevation forest	49.1010°N, 121.9210°W	*Rubus armeniacus*	Aug 31, Sep 7	3 (3 ♂)	G1
			Sambucus racemosa	Aug 1	3 (1 ♀, 2 ♂)	G1
	Wetland	49.0982°N, 122.0209°W	*Rubus armeniacus*	Aug 31	3 (2 ♀, 1 ♂)	G1
			Sambucus racemosa	Jun 25	3 (1 ♀, 2 ♂)	G1
	Low-elevation forest	49.2388°N, 122.5758°W	*Rubus idaeus*	Jul 16	3 (3 ♀)	G1
			Sambucus racemosa	Jul 9	3 (3 ♀)	G1
	Semi-urban park	49.1205°N, 123.0651°W	*Rubus armeniacus*	Aug 20	3 (3 ♀)	G1
			Sambucus racemosa	Jun 25, Jul 2	3 (1 ♀, 2 ♂)	G1
	Community garden	49.0352°N, 122.2448°W	*Sambucus racemosa*	Jun 26	3 (1 ♀, 2 ♂)	G1
			Rubus armeniacus	Oct 2	2 (1 ♀, 1 ♂)	G1
US (WA), 2020	Low-elevation forest	48.9163°N, 122.6824°W	[*V. mandarinia* trap]	Oct 10	1 (1 ♀)	G1
US (WA), 2021	Riparian hedgerow	48.9899°N, 122.4181°W	*Rubus armeniacus*	Sep 25	3 (3 ♀)	G1

Author contributions

EHB: manuscript preparation, editing, revising; project administration, funding acquisition; DB: manuscript preparation, revisions, live specimen collection; PS: manuscript preparation, live specimen collection, colony maintenance; PKA: manuscript preparation, review, editing, providing specimens for haplotyping; RSJ and EM: manuscript preparation, DNA extraction, sequencing; KMD: manuscript preparation, editing, revisions; CL: manuscript preparation, collecting and providing specimens for identification and sequencing; CHL: manuscript preparation, molecular identification; MB: manuscript preparation, molecular and morphological identification, specimen curation.

Results and discussions

Both *L. japonica* and *G. brasiliensis* were found in Whatcom County, WA (Fig. 3). There are indications that the two species co-occur regularly, as was reported in coastal British Columbia (Abram et al. 2020, 2022a). No species of *Asobara* Förster (Hymenoptera: Braconidae), which are common parasitoids of Drosophilidae, were found in Washington. However, the two Figitidae species were the targets of this limited survey, and more extensive surveys may reveal the presence of *Asobara* species; for example, *Asobara* cf. *rufescens* (Förster), was found associated with *D. suzukii* in nearby British Columbia (Abram et al. 2020, 2022a).

The COI sequences for *G. brasiliensis* were identical to the those generated from specimens collected in eastern Fraser Valley region of British Columbia in 2019 (Abram et al. 2020). The sequences for both populations place them in the G1 group. All 33 *G. brasiliensis* collected from British Columbia in 2020 and sequenced in this study were also determined to belong to the G1 group (Table 1), supporting the idea that this is the only (or at least the predominant) group present in the areas of North America where *G. brasiliensis* has been detected to date. The G1 *G. brasiliensis* group has been approved for release in the US by the USDA APHIS permitting process, primarily based on its higher degree of host specificity compared to other *G. brasiliensis* groups.

Phylogenetic analysis indicated that the *L. japonica* sequence clustered with two *L. japonica* sequences in the DROP database (DROP_sequence_id 448 - refers to voucher_id: 107, GenBank ID: MK268803, and DROP_sequence_id 445 - refers to voucher_id: 106, GenBank ID: MK268802). The voucher specimens which yielded the *L. japonica* sequences in a previous project (Buffington et al. 2020) were compared to the specimens in the current study, and determined to be the same species.

Detection of adventive, exotic parasitoids has been common in recent years, but in fact has a fairly long history as a means of establishment of these biological control agents (Weber et al. 2021). Several examples are known from Washington's tree fruit pest and natural enemy complex. Arguably, the first example was the parasitoid *Ascogaster quadridentatus* Wesmael (Hymenoptera: Braconidae), a European species which attacks codling moth (*Cydia pomonella* L.) (Lepidoptera: Tortricidae). The first adventive population of *A. quadridentatus* in the US was found in the eastern states in the early 1900s (Unruh 1998), and it became widely established there. However, once detected, its spread in the Pacific Northwest was by targeted release campaigns in Washington (Johansen 1957) and British Columbia (McLeod 1951; McLeod 1954). It was well established by the late 1990s and was the subject of several post-release studies (Reed-Larsen and Brown 1990; DeLury et al. 1999).

In the 1990s, *Colpoclypeus florus* (Walker) (Hymenoptera: Eulophidae), a European leafroller parasitoid, was found in Washington (Brunner 1996), likely well after it had been established for many years. At the time of its identification, it was already considered to provide substantial levels of biological control, and became the centerpiece of further studies to increase its impact through conservation (Pfannenstiel et al. 2010; Unruh et al. 2012). In 2014, *Anagyrus schoenherri*, (Westwood) (Hymenoptera: Encyrtidae) a parasitoid of apple mealybug, *Phenacoccus aceris*, (Signoret) (Hemiptera: Pseudococcidae) was identified for the first time in North America (Bixby-Brosi et al. 2017). This European species was well known from this cosmopolitan pest elsewhere, but had gone unnoticed in the US; however, it is likely this parasitoid was present as early as 2007 in Washington (EHB, unpublished). The discovery of the Asian egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), which attacks

Figure 3. Map of *G. brasiliensis* and *L. japonica* detections in northwestern Washington State, US in 2020–2021. Markers with both colors indicate both species were found at that site. Symbols represent presence or absence and are not indicative of relative abundance. Map tiles by Stamen Design, under CC BY 3.0. Data by OpenStreetMap, under ODbL.

the invasive pest brown marmorated stink bug, *Halyomorpha halys* Stål (Heteroptera: Pentatomidae), was made for the first time in North America in Maryland in 2014 (Talamas et al. 2015) and shortly thereafter in Washington state (Milnes et al. 2016), Oregon (Hedstrom et al. 2017), California (Lara et al. 2019), and a host of other states (Northeastern IPM Center 2022). Unlike the detections of *A. quadridentata* and *C. florus*, there was a nationwide effort (Ludwick et al. 2020) involving multiple laboratory groups to detect *T. japonicus*, greatly improving its likelihood of detection.

Similarly, the detection of the two Asian parasitoids of *D. suzukii* reported in the current study was greatly enhanced by communication and collaboration among scientists from the US, Canada, Europe, and Asia (Abram et al. 2022b). Once the first North American detections were made in British Columbia, it was highly probable that they would also occur in the contiguous habitat across the border of the US in northwestern Washington. While the exact dates and original location(s) of introduction(s) can never be known with certainty, it is likely that intensive effort and communication resulted in detection in relatively few years after introduction. Even the small geographic range extension we describe here, because it extends across an international border, has significant implications for how biological control against *D. suzukii* in the US will proceed in the coming years.

The adventive establishment of these two parasitoid species may have both positive and negative aspects for North American ecosystems. In particular, the presence of *L. japonica*, with its broader host range (Girod et al. 2018b; Daane et al. 2021), may cause non-target effects, in this case on native Drosophilidae, that regulation around the introduction of entomophagous biological control agents is expressly designed to prevent. Its broader host range may make *L. japonica* more likely to sustain itself in the invaded region in the event of local or temporary scarcity of *D. suzukii*, and also has the potential to mediate apparent competition between *D. suzukii* and other Drosophilidae. While the host range of *L. japonica* made it a less suitable candidate for intentional release, the two species of figitids could provide complementary suppression of *D. suzukii* in areas where they co-occur (Wang et al. 2019).

All finds of *G. brasiliensis* and *L. japonica* in North America to date have been in moist, mild-winter coastal regions of the Pacific Northwest (Abram et al. 2022a). However, the primary tree fruit growing regions in Washington and British Columbia, as well as a growing blueberry industry in Washington, are in the semi-arid regions in the rain shadow of the Cascade mountains. Having two parasitoid species or populations increases the likelihood that they can become established. Currently, the permitting process will allow confirmed G1 *G. brasiliensis* colonies to be spread throughout the US, which is part of an ongoing national project. However, Washington populations of *L. japonica* cannot be further disseminated out of Washington.

The co-occurrence of *G. brasiliensis* and *L. japonica* in both British Columbia and Washington may shed more light on parasitoid impacts, complementarity-competition and non-target effects on native Drosophilidae (Wang et al. 2019). This will enable researchers to verify the results and predictions of laboratory experiments in field settings, and provide a deeper understanding of the host-specificity testing process. Recently developed molecular diagnostic techniques (Gariepy et al. 2019; Hepler et al. 2020) may be employed to overcome the inability to make direct observations and provide a more comprehensive view of host-parasitoid interactions.

Lastly, adventive populations may serve as a reservoir that can be accessed if laboratory colonies fail. Rearing parasitoids in quantities large enough for mass release, which is currently planned in locations across the US, will pose some significant challenges, including the cost of insectary space, labor and materials. Currently, *G. brasiliensis* are most easily reared on *D. suzukii* larvae in a fruit host, which greatly increases the cost of rearing over artificial diet. Field populations of *G. brasiliensis* in the US have the potential to act as a readily accessible source of genetic variation (assuming adaptation occurs *in situ*) that could potentially improve the success of releases in other areas of the country by avoiding bottlenecks typically associated with laboratory rearing (Hopper et al. 1993).

Conclusions

The relatively widespread nature of the populations of G1 group of *G. brasiliensis* and *L. japonica* in British Columbia and the novel, although limited, knowledge of its distribution in Washington suggest it is well established in the region. More extensive surveys in Washington are needed to determine the extent of establishment. Part of the survey process must include group determination for *G. brasiliensis*, which can inform the pathway and number of introductions. Ultimately, the goal for researchers must be determining not just the presence, but the impact of these species on the target pest. If the parasitoids are susceptible to insecticides, they may play a limited role in directly controlling *D. suzukii* that reproduce within intensively managed cropping systems. However, they may play a significant role in suppressing the populations of *D. suzukii* in wild hosts, thus reducing immigration into crop fields.

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