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Research Article

Widespread establishment of adventive populations of *Leptopilina japonica* (Hymenoptera, Figitidae) in North America and development of a multiplex PCR assay to identify key parasitoids of *Drosophila suzukii* (Diptera, Drosophilidae)

Tara D. Gariepy[1], Paul K. Abram[2], Chris Adams[3], Dylan Beal[4], Elizabeth Beers[4], Jonathan Beetle[5], David Biddinger[6], Gabrielle Brind'Amour[7], Allison Bruin[1], Matthew Buffington[8], Hannah Burrack[9], Kent M. Daane[10], Kathleen Demchak[11], Phillip Fanning[12], Alexandra Gillett[5], Kelly Hamby[13], Kim Hoelmer[14], Brian Hogg[15], Rufus Isaacs[9], Ben Johnson[12] Jana C. Lee[16], Hannah K. Levensen[17], Greg Loeb[7], Angela Lovero[5], Joshua M. Milnes[18], Kyoo R. Park[19], Patricia Prade[20], Karly Regan[11], Justin M. Renkema[21], Cesar Rodriguez-Saona[20], Subin Neupane[22], Cera Jones[22], Ashfaq Sial[22], Peter Smythman[4], Amanda Stout[14], Steven Van Timmeren[9], Vaughn M. Walton[19], Julianna K. Wilson[9], Xingeng Wang[14]

1 *Agriculture and Agri-Food Canada, London Research and Development Centre, 1391 Sandford Street, London, Ontario, Canada*
2 *Agriculture and Agri-Food Canada, Agassiz Research and Development Centre, 6947 Highway 7, Agassiz, British Columbia, Canada*
3 *Mid-Columbia Agricultural Research and Extension Center, 3005 Experiment Station Dr, Hood River, Oregon, USA*
4 *Washington State University, Tree Fruit Research and Extension Center, 1100 N Western Ave, Wenatchee, Washington, USA*
5 *NJ Department of Agriculture, Phillip Alampi Beneficial Insect Laboratory, 20 Cosey Road, West Trenton, New Jersey 08628, USA*
6 *Penn State College of Agricultural Sciences, Department of Entomology, Fruit Research and Extension Center, 290 University Drive, Biglerville, Pennsylvania, USA*
7 *Cornell University, Department of Entomology, Geneva, New York, USA*
8 *Systematic Entomology Laboratory, ARS/USDA c/o Smithsonian Institution, National Museum of Natural History, Washington, DC, USA*
9 *Department of Entomology, Michigan State University, East Lansing, MI 48824, USA*
10 *Department of Environmental Science, Policy and Management, University of California, Berkeley, California, USA*
11 *Penn State College of Agricultural Sciences, Department of Plant Science, 102 Tyson Building, University Park, Pennsylvania, USA*
12 *University of Maine, School of Biology and Ecology, 5722 Deering Hall, Orono, Maine, USA*
13 *University of Maryland, Department of Entomology, 4291 Fieldhouse Drive, College Park, Maryland, USA*
14 *USDA-ARS, Beneficial Insects Introduction Research Unit, 501 South Chapel Street, Newark, Delaware, USA*
15 *USDA-ARS Invasive Species and Pollinator Health, Albany, California, USA*
16 *USDA ARS Horticultural Crops Disease and Pest Management Research Unit, 3420 NW Orchard Ave, Corvallis, Oregon, USA*
17 *North Carolina State University, Department of Entomology and Plant Pathology, Campus Box 7613, Raleigh, North Carolina, USA*
18 *Washington State Department of Agriculture, Plant Protection Division, 21 North 1st Avenue, Suite 103, Yakima, Washington, USA*
19 *Oregon State University, Department of Horticulture, College of Agricultural Sciences, 2750 SW Campus Way, Corvallis, Oregon, USA*
20 *Rutgers, P.E. Marucci Center, 125A Lake Oswego Drive, Chatsworth, New Jersey, USA*
21 *Agriculture and Agri-Food Canada, London Research and Development Centre, Vineland Campus, 4890 Victoria Ave N, Vineland Station, Ontario, Canada*
22 *University of Georgia, 463 Biological Sciences Building, 120 Cedar St., Athens, Georgia, USA*
Corresponding author: Tara D. Gariepy (tara.gariepy@agr.gc.ca)

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Abstract

In recent years, there has been an increase in the adventive establishment and spread of parasitoid wasps outside of their native range. However, lack of taxonomic tools can hinder the efficient screening of field-collected samples to document the establishment and range expansion of parasitoids on continent-wide geographic scales. Here we report that *Leptopilina japonica* (Hymenoptera, Figitidae), a parasitoid of the globally invasive fruit pest *Drosophila suzukii* (Diptera, Drosophilidae), is now widespread in much of North America despite not having been intentionally introduced. Surveys in 2022 using a variety of methods detected *L. japonica* in 10 of 11 surveyed USA States and one Canadian Province where it was not previously known to occur. In most surveys, *L. japonica* was the

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most common species of *D. suzukii* parasitoid found. The surveys also resulted in the detection of *Ganaspis* cf. *brasiliensis* (Hymenoptera, Figitidae), the recently-released biological control agent of *D. suzukii*, in six USA States where it had not previously been found. These new detections are likely a result of intentional biological control introductions rather than spread of adventive populations. A species-specific multiplex PCR assay was developed as a rapid, accurate and cost-effective method to distinguish *L. japonica*, *G.* cf. *brasiliensis*, the closely-related cosmopolitan parasitoid *Leptopilina heterotoma* (Hymenoptera, Figitidae) and other native parasitoid species. This dataset and the associated molecular tools will facilitate future studies of the spread and ecological impacts of these introduced parasitoids on multiple continents.

Key words: Adventive establishment, DNA barcoding, *Drosophila suzukii*, *Ganaspis brasiliensis*, Hymenoptera, molecular diagnostics, multiplex PCR, spotted-wing drosophila, unintentional biological control

Introduction

The rate of global introductions of non-native insect species due to human activities continues to accelerate (Seebens et al. 2017; Seebens et al. 2021). Many of these introductions result in widespread negative environmental, human health and economic impacts (Bellard et al. 2016; Diagne et al. 2021). A minority of these introductions may have some positive consequences or a mixture of positive and negative effects (Schlaepfer et al. 2011; Vimercati et al. 2020; Sax et al. 2022). The potential for desirable or mixed effects is particularly likely with unintentionally introduced parasitoids and predators of invasive insects (Roy et al. 2011; Weber et al. 2021). While it is increasingly recognised that unintentional introductions of species occupying higher trophic levels are probably common (Weber et al. 2021), many are likely undetected and, as such, there are relatively few examples that have been well-documented on continental scales.

Parasitoid wasps are one of the most biodiverse groups of animals on the planet (Forbes et al. 2018) and are the most common group of biological control agents used to target invasive insects (Heimpel and Mills 2017). Parasitoid wasps have frequently been intentionally introduced as biological control agents worldwide, successfully controlling numerous invasive pest species in forestry, agriculture, and conservation contexts (Mason 2021). Host range is the strongest filter used to select parasitoid species for modern importation biological control programmes. To minimise the potential for non-target ecological impacts, only natural enemies with relatively narrow host ranges are selected from the native range of an invasive pest (Bigler et al. 2006; Hoddle et al. 2021). However, parasitoids from the native range of an invasive pest, even those with broader host ranges that may be less suitable for biological control programmes, can occasionally establish themselves in regions previously invaded by their host. These unintentional introductions can occur outside the scope of the standard biological control regulatory process (e.g. Frewin (2010); Talamas et al. (2015); Mason et al. (2017); Peverieri et al. (2018); Weber et al. (2021)). There is growing interest in documenting the spread of unintentionally introduced parasitoids and assessing the balance of their positive and negative ecological and environmental impacts (Mason et al. 2017; Weber et al. 2021).

One major challenge for documenting the spread of introduced parasitoids in their new geographic ranges is a taxonomic impediment. For many parasitoid

groups, the native versus non-native parasitoid fauna itself may not be entirely known and may be difficult to distinguish based on morphology. Further, it may be unfeasible or inefficient for the limited number of taxonomic experts on a given group of parasitoids to provide identification services for numerous research teams conducting large-scale surveys. The use of molecular tools, including species-specific PCR primers, for parasitoid species identification can help alleviate this problem (Gariepy et al. 2005; Traugott et al. 2006; Gariepy et al. 2007, 2008; Rugman-Jones et al. 2011, 2020; Gariepy and Messing 2012; Shariff et al. 2014; Furlong 2015; Shimbori et al. 2023). However, their development requires DNA sequences that are tied to authoritatively identified specimens (Lue et al. 2021; Shimbori et al. 2023), which is often not the case, given that many of the world's parasitoid fauna remain undescribed (Forbes et al. 2018). Fortunately, when an economically-important invasive pest is the focus of an importation biological control programme, the specimens and taxonomic knowledge may be available to facilitate the development of molecular diagnostic tools that can be used to document the spread and impact of unintentionally introduced parasitoids.

These dynamics are currently playing out on a global scale for parasitoids of spotted-wing drosophila, *Drosophila suzukii* Matsumura (Diptera, Drosophilidae), an invasive vinegar fly that has become a significant pest in all major fruit production areas in the Americas, Europe and parts of Africa (Asplen et al. 2015; Tait et al. 2021). Although the first report of *D. suzukii* establishment outside of its native range was in Hawaii in 1980 (Kaneshiro 1983), subsequent spread was not reported until 2008 when *D. suzukii* was found in California (Hauser 2011). By 2011, populations of *D. suzukii* were well-established in fruit-growing areas in western and eastern North America (Tait et al. 2021), presumably as a result of movement and spread of already-established populations from the western US (Fraimout et al. 2017). Surveys for parasitoids soon after the invasion of North America and Europe did not detect any specialised parasitoids of *D. suzukii* (Lee et al. 2019; Wang et al. 2020). Foreign exploration for parasitoids of *D. suzukii* in its native range of Asia documented a total of 21 parasitoid species (summarised in Wang et al. 2020), with three dominant parasitoids attacking *D. suzukii* larvae: *Asobara japonica* Belokobylskij (Hymenoptera, Braconidae), *Ganaspis* cf. *brasiliensis* Ihering and *Leptopilina japonica* Novković & Kimura (Hymenoptera, Figitidae) (Mitsui et al. 2007; Daane et al. 2016; Girod et al. 2018a; Giorgini et al. 2019). One strain of *G.* cf *brasiliensis* (called "G1") was selected and approved for intentional biological control releases in 2021 in the USA and Italy because of its specificity to *D. suzukii* (Biondi et al. 2021; Daane et al. 2021; Lisi et al. 2021; Fellin et al. 2023). Another genetically distinct lineage of *G.* cf. *brasiliensis* (referred to as G3) co-occurs with G1 in East Asia; however, the two strains are reproductively incompatible (Seehausen et al. 2020) and will be described as two distinct species (M. Buffington, in prep). *Ganaspis* cf. *brasiliensis* G3 has a broader host range that includes other *Drosophila* species and was, therefore, not considered for intentional release (Girod et al. 2018a; Giorgini et al. 2019; Seehausen et al. 2020; Daane et al. 2021). Similarly, approval for the release of *L. japonica* and *A. japonica* was not pursued because their host range included several species of Drosophilidae other than *D. suzukii* (Girod et al. 2018b; Giorgini et al. 2019; Daane et al. 2021; Seehausen et al. 2022).

However, just prior to intentional releases of *G.* cf. *brasiliensis* G1, field surveys in the Pacific Northwest of North America revealed the presence of adventive (pre-

sumed to be accidentally introduced) populations of *L. japonica* (as early as 2016) and *G.* cf. *brasiliensis* G1 (in 2019) in British Columbia, Canada (Abram et al. 2020). By 2020, it was clear that both species were well-established in British Columbia, parasitising *D. suzukii* in diverse habitats (Abram et al. 2022a). Both parasitoid species were subsequently found in Washington, USA in 2020 and 2021, respectively (Beers et al. 2022). Similarly, adventive populations of *L. japonica* were discovered in northern Italy in 2019 (Puppato et al. 2020) and Germany in 2021 (Martin et al. 2023). These observations suggest that *L. japonica* and *G.* cf. *brasiliensis* may be rapidly spreading to areas outside their native range due to unintentional introductions. In North America, however, the distribution of adventive populations of these parasitoids outside of the Pacific Northwest was unknown.

During 2022, multiple research groups independently initiated new surveys to document the parasitoid community of *D. suzukii* as part of the intentional release programme for *G.* cf. *brasiliensis* G1. However, given the large number of samples involved in these surveys, efficiently documenting the presence of *L. japonica*, in particular, was challenging and time-consuming because it is exceedingly difficult to distinguish from related native fauna, especially the cosmopolitan species *L. heterotoma* Thompson (Abram et al. 2020; Abram et al. 2022b). In fact, before having access to specimens of *L. japonica* from Asia, the leading taxonomic expert on Figitidae in North America misidentified the first *L. japonica* found in North America as *L. heterotoma* (Abram et al. 2020).

In this study, we consolidated the findings of research groups surveying for parasitoids of *D. suzukii* in 12 USA States and one Canadian Province. We used this combined dataset to assess how geographically widespread *L. japonica* and *G.* cf. *brasiliensis* G1 are in the surveyed areas of North America. In addition, to address the difficulty of parasitoid identification in these and future surveys, DNA barcodes were generated from field-collected specimens to confirm the identity and to generate an authoritative DNA barcode library for those species likely encountered in *D. suzukii* parasitoid surveys. Then, a single-step multiplex PCR assay was developed for *G*. cf. *brasiliensis* G1, *L. japonica* and *L. heterotoma* to facilitate screening of large numbers of field-collected samples for the presence of adventive and intentionally introduced parasitoid species. We anticipate that continent-wide survey data, combined with new molecular diagnostic tools, will help provide information for future strategies to release and re-distribute *D. suzukii* parasitoids and will serve as a baseline for measuring the balance of positive and negative ecological effects of unintentionally introduced parasitoid species.

Methods

Geographic occurrence and species composition of parasitoids associated with *Drosophila suzukii*

Surveys were initiated in 2022 using a variety of methodologies throughout western and eastern regions in North America to characterise the community of parasitoids associated with Drosophilidae, focusing on *D. suzukii*. In most locations, direct sampling of ripe fruits was the primary collection method in the USA (California, Delaware, Maryland, Michigan, New Jersey, New York, North Carolina, Oregon, Pennsylvania, Washington) and Canada (Ontario); however, deployment of *D. suzukii*-baited sentinel fruits in the field was also used to detect the presence

of Asian parasitoids in Georgia, Maine, New Jersey, North Carolina and Oregon. In addition, opportunistic sampling of adult Figitidae retrieved as by-catch from a variety of other traps deployed for monitoring *D. suzukii* were collected where available (Ontario, North Carolina and Washington); however, these traps are not specific in terms of capturing Asian parasitoids of *D. suzukii* and are likely to also contain native or cosmopolitan figitids associated with other drosophilids. Fruit collections and deployment of fruit-baited sentinel traps in the USA took place in the vicinity of *G.* cf. *brasiliensis* G1 release sites in 2022. *Ganaspis* cf. *brasiliensis* G1 was not released in Ontario, Canada. Detailed methods for each collection type are listed below. For each collection event, GPS coordinates (approximated to preserve land-owner privacy) were recorded and associated with individual wasp specimens retrieved so that the distribution of Asian parasitoids of *D. suzukii* could be assessed (see Suppl. material 1 for collection information). It is important to note that the dataset presented here has been generated by several groups and there are some modest differences in methodology between groups; as such, our intent is to report on the presence or absence of Asian parasitoids of *D. suzukii* in surveyed locations, as opposed to reporting detailed datasets on temporal parasitism and parasitoid species composition.

Direct sampling of fruit from the field

Following the recommended methodology described in Abram et al. (2022b), parasitoid adults were obtained through collection and rearing of fly puparia from ripe fruits from wild and cultivated hosts from one Canadian Province and 10 US States (Table 1). Sampling of fruits from host plants was performed at focal sites in most locations in 2022 at intervals of 7–14 days over a period of approximately 3–4 months, based on the temporal patterns of ripening fruits throughout the growing season. However, intensive sampling over a shorter period (e.g. 1 month) on the same host plant was performed at some locations. Collection information is provided in Suppl. material 3, including locations, dates and host plants sampled. The sampling effort (number of sites, sampling period) and number of specimens obtained from each State/Province varied (Tables 1, 2) and, as such, it was not possible to quantitatively compare the prevalence of each species between locations; however, the data were analysed from a more descriptive standpoint.

Fruit was incubated in ventilated plastic containers as described by Abram et al. (2022b) to allow development of drosophilid larvae and parasitoids. Briefly, collected fruits were placed in a plastic container (500–750 ml volume), typically on a raised metal grid made of wire or hardware cloth (with an appropriate-sized grid such that fruit do not fall through the grid, but fly larvae can easily drop out of the fruit into the bottom of the container). Absorbent paper or cotton pads were placed in the bottom of the container to absorb fruit juices as the fruits decayed and to provide a pupation substrate for larvae. Containers were closed with a ventilated lid (ventilation hole was approx. 5–7 cm in diameter, covered with fine mesh cloth). Samples were stored in a growth chamber at 21–25 °C, 16:8 (L:D) light cycle and 50–55% relative humidity (RH) or kept under air-conditioned room temperatures (20–23 °C) with natural light accessible from the windows. Containers were checked every 2–3 days and drosophilid puparia were collected and reared either in ventilated Petri-dishes or individual 1.5 ml centrifuge tubes. Emergence was monitored every 2–3 days and the number of parasitoid wasps was recorded.

Table 1. Overview of parasitoid collections from direct sampling of fruit in North America in 2022, including host plant species from which parasitoids emerged.

Country	Province / State	#sites	#Parasitoids	Collection Period	Host Plant(s)
Canada	Ontario	7	39	June – September	*Rubus occidentalis, Sambucus nigra, Cornus obliqua, Rhamnus cathartica*
USA	California	2	34	June – November	*Rubus idaeus, Rubus ulmifolius*
	Delaware	3	328	June – October	*Rubus allegheniensis*, *Phytolacca americana, Elaeagnus umbellata, Persicaria perfoliata, Prunus serotina, Rubus phoenicolasius*
	Maryland	3	1198	August – October	*Elaeagnus umbellata, Rubus* spp., *Rubus idaeus, Lonicera* spp., *Phytolacca americana*
	Michigan	16	82	July – August	*Vaccinium corymbosum*., *Phytolacca americana, Rubus* spp., *Sambucus canadensis*., *Lonicera* spp.
	New Jersey	2	7	July – September	*Gaylussacia* spp., *Vaccinium* spp.
	New York	1	3	August	*Rubus idaeus*
	North Carolina	4	21	July – October	*Rubus idaeus*, *Rubus* subgenus *Rubus* (blackberry), *Phytolacca americana, Celastrus orbiculatus*
	Oregon	6	252	July – October	*Rubus armeniacus; Rubus idaeus*
	Pennsylvania	2	234†	August – October	*Ribes rubrum*, *Rubus* subgenus *Rubus* (blackberry), *Lonicera maackii, Phytolacca americana, Rubus* spp., *Elaeagnus umbellata*, *Vaccinium corymbosum*
	Washington	24	794	August	*Rubus armeniacus, Prunus avium*

† A total of 234 parasitoids were obtained; however, only 126 were provided for morphological or molecular identification.

Table 2. Number of parasitoids collected and identified (based on morphology and/or molecular methods) from ripe fruit collections in North America in 2022. Lj = *Leptopilina japonica*, Gb = *Ganaspis* cf. *brasiliensis* G1, Ar = *Asobara* cf. *rufescens*, Pv = *Pachycrepoideus vindemiae*, Td = *Trichopria drosophilae*, No ID = unidentified.

Country	Province/State	Total # Parasitoids	# Barcoded / PCR	Parasitoid species identification					
				Lj	*Gb*	*Ar*	*Pv*	*Td*	No ID
Canada	Ontario	39	39	38	0	0	0	0	1
USA	California†	34	0	0	0	0	0	34	0
	Delaware†	328	26	260	64	4	0	0	0
	Maryland†	1198	96	1190	8	0	0	0	0
	Michigan	82	82	82	0	0	0	0	0
	New Jersey	7	7	3	2	0	0	0	2
	New York	3	3	3	0	0	0	0	0
	North Carolina	21	14	21	0	0	0	0	0
	Oregon†	252	45	233	5	0	14	0	0
	Pennsylvania†	126††	116	110	16	0	0	0	0
	Washington†	794	34	696	95	3	0	0	0
	Total	**2884**	**462**	**2636**	**190**	**7**	**14**	**34**	**3**

† States from which some specimens were identified morphologically, typically with a subsample of Figitidae identified using molecular approaches (DNA barcoding and/or multiplex PCR); ††An additional 108 specimens were collected, but were not provided for morphological or molecular identification.

Containers were also checked every 2–3 days for emergence of parasitoids that pupated within the fruit. Emerged wasps were placed directly into 95% ethanol or killed by freezing at -20 °C prior to placing in 95% ethanol. However, samples from Michigan were typically captured on sticky cards that were placed inside the container and these parasitoids were soaked in Histoclear (National Diagnostics,

Atlanta, Georgia) to loosen the glue, carefully removed with a dissecting needle or forceps and then put into 95% ethanol. When many parasitoids were collected, subsamples were DNA barcoded (see Table 2) and the remaining wasps were identified, based on morphological characteristics (as per Abram et al. (2022b)).

Drosophila suzukii-baited sentinels

The exact set-up of sentinel baits differed somewhat amongst the teams deploying the sentinels, but were based on the recommendations described by Abram et al. (2022b) and are summarised below. In general, 10 blueberries and 2.5 cm thick in-peel slices of banana were dipped in a 5% bleach solution, allowed to off-gas and then sprinkled with yeast to reduce mould growth in the field. The fruit was then exposed to *D. suzukii* adults for 48 hours to allow infestation to occur. Infested fruit was placed in 250 ml plastic containers (with lids) with 16 holes (5 mm diameter) punched equally around the outside perimeter. The plastic container was placed in a Trécé Pherocon VI Delta Trap (Great Lakes IPM, Vestaburg, Michigan, USA) and suspended from trees along the edges of *D. suzukii*-susceptible crops, approximately 1.5 m above the ground. Sentinel traps were left in the field for 7 days before being returned to the lab. Fruit from inside the sentinel traps was transferred to 8 × 8 × 2 cm wire mesh berry holders (0.5 cm grid) and placed in a 500 ml plastic container on top of a folded piece of paper towel and dental wicking to absorb moisture as the fruit broke down. Once re-collected from the field, contents in the traps were incubated in the lab as described above for field-collected fruit. Emerged wasps were collected, recorded and preserved in 95% ethanol for molecular identification. An overview of sentinel trapping is presented in Table 3 and exposure information is provided in Suppl. material 1.

Opportunistic sampling of by-catch from other trap types

Apple cider vinegar (ACV) traps were deployed in non-crop habitats bordering commercial berry sites in three locations in south-western Ontario (Suppl. material 1). Homemade traps were constructed from 1 litre clear plastic containers (Richards Packaging, Mississauga, Ontario, Canada) and eight entry holes (1.8 cm diameter) were drilled around the upper portion of the jar, leaving an area without holes to facilitate emptying of the jar contents. Holes were screened with fibreglass drywall tape (2.5 × 2.5 mm openings) (Sheetrock, CGC Corp., Chicago, Illinois, USA) to allow drosophilid flies and parasitoids to enter, but to exclude larger insects. The bottom half of each jar was covered in red tape (Cantech Industries Inc., Johnson City, Tennessee, USA) to increase attraction and a red plastic plate (22 cm diameter) was affixed to the lid of the jar as a rain barrier and an eyelet screw was placed through the jar lid and plate to facilitate hanging using twist ties. Traps were hung from metal rods placed in the ground adjacent to berry crops. Approximately 250 ml of an ACV-ethanol mixture was added (1 part 95% ethanol to 9 parts ACV). Trap contents were collected weekly from 8 July to 18 August 2022 and were visually inspected for the presence of adult figitids; specimens were retrieved and stored in 95% ethanol for molecular identification (Table 3).

Wine-vinegar traps were deployed to monitor for *D. suzukii* in Washington, using the PHEROCON SWD cup trap (Trécé Inc., Adair, Oklahoma, USA) baited with a wine-vinegar bait (Franzia Crisp White Wine and Western Family Apple

Table 3. Parasitoids of Drosophilidae collected from apple cider vinegar (ACV) traps, Scentry traps, ACV/wine traps and *Drosophila suzukii* (Ds)-baited sentinel fruit. All parasitoid identifications were done by sequencing the COI DNA barcode region. Lj = *Leptopilina japonica*, Gb = *Ganaspis* cf. *brasiliensis* G1, Lh = *Leptopilina heterotoma*, Ll = *Leptopilina leipsi*, Lm = *Leptopilina maia*, No ID = unidentified.

Country	Province/State	Collection type	# Parasitoids collected	Collection Period	Parasitoid species composition (%)
Canada	Ontario	ACV traps	36	Jul–Aug	Lj (100%)
		Scentry traps	15	May–Jul	Lj (53%), Ll (33%), Lh (7%), Lm (7%)
USA	Washington	Scentry traps	6	Jul–Oct	Lj (33%), Lh (33%), Gb (33%)
		ACV/Wine traps	8	Jun–Sep	Lj (50%), Lh (50%)
	North Carolina	Scentry traps	9	Aug–Nov	Lj (33%), Lh (33%), Lm (33%)[†]
		Ds-baited sentinels	3	Jul	No identification (failed to amplify and sequence)
	Oregon	Ds-baited sentinels	3	Jun–Sep	Lj (100%)
	New Jersey	Ds-baited sentinels	17	Aug–Sep	Lj (94%), Gb (6%)
	Maine	Ds-baited sentinels	94	Sep	Lj (100%)
	Georgia	Ds-baited sentinels	3	Sep–Oct	Lj (67%), Gb (33%)

[†] Percentages reflect three identified specimens. Six specimens did not yield DNA sequences or PCR results and could not be identified.

Cider Vinegar, 50:50 mix, plus ~ 1 ml of Palmolive Pure and Clear Unscented dish soap per litre of wine/vinegar mix). Traps were placed in a wild host plant adjacent to cherry orchards and the contents were checked weekly throughout the growing season. Adult Figitidae found in the traps were retrieved and stored in 95% ethanol for subsequent identification using molecular techniques (Table 3).

Scentry traps consisted of homemade jar traps (as described above for the ACV traps) baited with a commercial Scentry lure (Scentry Biologicals, Billings, Montana, USA) and a drowning fluid (water, dish soap and sodium benzoate or a 50:50 mix of antifreeze and water). Traps were placed in commercial berry sites (Ontario and North Carolina) or urban parklands with susceptible host plants (Washington) and contents were collected every 4–16 days from 15 May–15 July 2022 (Ontario), 26 July–17 October 2022 (Washington) and from 25 August–3 November 2022 (North Carolina). Trap contents were visually inspected for the presence of adult Figitidae. Specimens were retrieved and stored in 95% ethanol for identification using molecular techniques (Table 3).

Molecular identification tools for parasitoids of *Drosophila suzukii*

DNA barcoding of specimens

To confirm the identity of specimens collected in the surveys described above, DNA barcoding was implemented to screen Figitidae collected from ripe fruit, sentinel fruit and other baited traps (n = 653). However, when a large number of parasitoids (> 100) was recovered from a given location, morphological characteristics were used to identify most specimens (Abram et al. 2022b) and only subsamples were barcoded. In addition to field-collected samples, 64 identified specimens from laboratory colonies maintained at the quarantine facility of the United States Department of Agriculture (USDA) Beneficial Insects Introduction Research Unit (BIIRU; Newark, Delaware, USA) were barcoded to serve as reference sequences. This included: 15 *G.* cf. *brasiliensis* G1 (originating from Japan), 10 *G.* cf. *brasiliensis* G3 (originating from China), 10 *L. japonica* (originating from China), 9

L. japonica (originating from South Korea) and 20 *A. japonica* (originating from South Korea). All wasps were freshly killed in 95% ethanol, except *L. japonica* from China, which were stored dry after the colony had collapsed and only dead dry wasps were available for molecular analysis. Details of DNA extraction, amplification and DNA sequencing methods are outlined in Suppl. material 2. Sequence data and trace files were uploaded to the Barcode of Life Datasystems (BOLD; www.boldsystems.org) in the Project "APSWD, Adventive populations of SWD parasitoids" (for field-collected specimens) and Project "VPDRS, Verified parasitoids of *Drosophila suzukii*" (for identified specimens from laboratory colonies); both of these databases are publicly available. DNA barcodes from field-collected samples were identified using the BOLD Identification Engine by searching the species-level DNA barcode records for a match. In addition, all material that was DNA-barcoded (653 field-collected specimens and 64 specimens obtained from BIIRU) have been deposited in the insect collection of the National Museum of Natural History (USNM; Smithsonian Institution, Washington, DC).

Development of multiplex PCR primers

To facilitate screening and identification of large numbers of samples for current and future collections, the DNA barcode dataset (generated from colony-reared and field-collected specimens) was used to develop PCR primers that can be used in multiplex to separate *L. japonica*, *G.* cf. *brasiliensis* G1 and *L. heterotoma*, without amplifying the other parasitoid species that may be encountered in similar habitats (e.g. ripe or rotting fruits, baited traps). The DNA barcode sequences that we generated for *A.* cf. *rufescens* Foerster (Hymenoptera: Braconidae), *A. japonica*, *L. leipsi* Lue, *L. maia* Lue, *L. heterotoma*, *L. japonica*, *G.* cf. *brasiliensis* G1 and *G.* cf. *brasiliensis* G3 were aligned in CODONCODE ALIGNER version 9.0.1 (Codon-Code Corporation, Centerville, Massachusetts, USA). Based on areas of sequence variation between the different species, a unique forward primer nested within the DNA barcode region was designed for *L. heterotoma*, *L. japonica* and *G.* cf. *brasiliensis* G1 that, when combined with the reverse primer HCO-2198 (Folmer et al. 1994), generates a unique fragment size diagnostic for each species (Table 4). Using all three forward primers and the reverse primer in a single multiplex PCR reaction would allow simultaneous screening of a sample for multiple species to reduce the number of PCR reactions required to identify a sample. Once putative primers were located, they were imported into Primer3Plus (Untergrasser et al. 2012) to confirm their suitability (i.e. stability, melting temperature, potential for hairpins, primer dimers) and determine the appropriate temperature for amplification of the desired PCR product.

Each multiplex PCR reaction was performed in a 25 µl volume containing 0.125 µl of Taq Platinum, 2.5 µl of 10× PCR buffer, 1.25 µl of 50 mM $MgCl_2$, 0.125 µl of 10 µM dNTPs (Invitrogen, Carlsbad, California, USA), 0.25 µl of each 10 µM forward primer (Gb1F-353, LjF-46 and LhF-212, respectively), 0.5 µl of 10 µM reverse primer (HCO-2198), 2 µl UltraPure BSA (50 mg/ml; Invitrogen, Carlsbad, California, USA), 16.75 µl ddH20 and 1 µl of template DNA. The BSA was added to enhance the specificity and efficiency of the multiplex assay to reduce non-specific binding, particularly for regions with moderate to high GC-rich sequences (Nagai et al. 1998; Markoulatos et al. 2002; Strien et al. 2013). Thermocycling conditions included initial denaturation at 94 °C

Table 4. Putative species-specific forward primers for *Ganaspis* cf. *brasiliensis* (G1; Gb1F-353), *Leptopilina japonica* (LjF-46) and *Leptopilina heterotoma* (LhF-212) and sequence length when used in combination with the universal reverse primer HCO-2198 (number in brackets refers to PCR product length when primer sequences are trimmed from both ends).

Species-specific Forward Primer	Primer Sequence (5'–3')	PCR product length when used with HCO-2198
Gb1F-353	CTAAATAAGTCCCACCCAGGAATC	332 bp (282 bp)
LjF-46	TGGGTTAAGATTCCTTGTTCGTAC	639 bp (589 bp)
LhF-212	CTTACAGTTCCTGATATAGCATTTCCA	473 bp (420 bp)

for 1 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 1 min and a final extension period of 5 min at 72 °C. PCR products were visualised with a QIAxcel Advanced automated capillary electrophoresis system (Qiagen, Hilden, Germany) using the DNA screening cartridge and method AL320. Results were scored with QIAXCEL SCREENGEL Software (version 1.2.0), samples with signal strength exceeding 0.1 relative fluorescent units were scored as positive and species identity was assigned, based on the PCR fragment size generated (332 bp for *G.* cf. *brasiliensis* G1, 639 bp for *L. japonica* and 473 bp for *L. heterotoma*).

The specificity of the multiplex PCR was tested with DNA from five specimens of each of the following species (obtained from field collections and/or laboratory colonies): *G.* cf. *brasiliensis* G1; *G.* cf. *brasiliensis* G3; *L. japonica* (South Korea); *L. japonica* (China); *L. heterotoma*; *L. leipsi*; *L. maia*; *A. japonica*; and *A.* cf. *rufescens*. In addition, all samples that were DNA barcoded (n = 653) were screened using the multiplex PCR assay and the identity was compared to the DNA barcode results to determine whether they were consistent.

Results

Geographic occurrence and species composition of parasitoids associated with Drosophila suzukii

Direct sampling of fruit from the field

Across a total of 70 sampling sites, 2884 parasitoids (including 2636 *L. japonica* and 190 *G.* cf. *brasiliensis* G1) were obtained for morphological and/or molecular identification from ripe fruit collections in the sampled locations in North America (10 US States and one Canadian Province) (Table 2). Asian parasitoids associated with *D. suzukii* were detected in all States/Provinces sampled, except California (Figs 1, 2, Table 2). *Leptopilina japonica*, which represented 91% of all parasitoids identified, was present in 10 of the 11 States/Provinces surveyed, where it was collected from 11 different host plant genera (Table 2; Suppl. material 2: table S2). *Ganaspis* cf. *brasiliensis* G1 represented 7% of all parasitoids identified and was found in six States from a total of six different host plant genera (Table 2; Suppl. material 2: table S2); all *G.* cf. *brasiliensis* G1 detections occurred following the intended release of this species in summer 2022 as a part of the biological control programme targeting *D. suzukii* in these States, except Washington, where it was already present prior to releases (Beers et al. 2022).

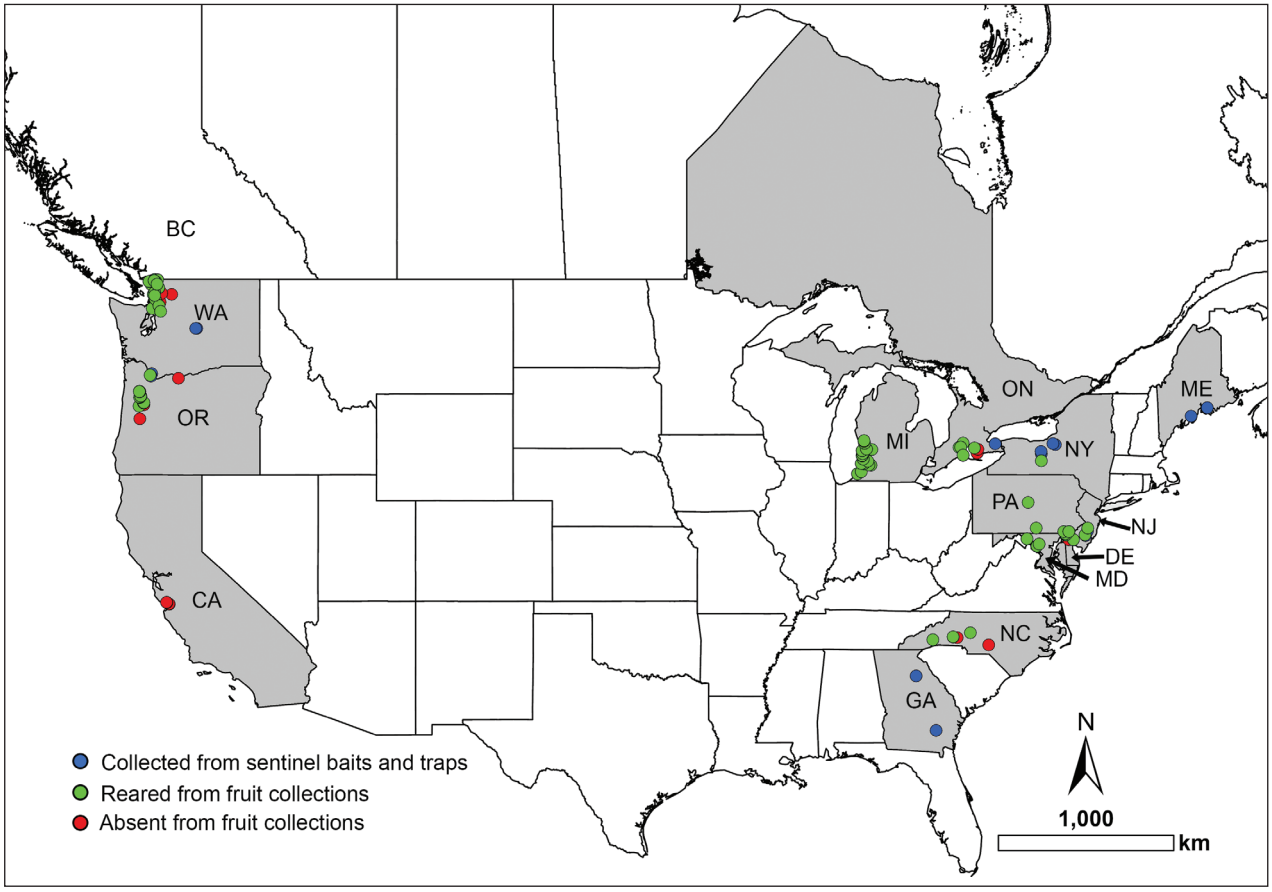

Figure 1. Detections of *Leptopilina japonica* in Canada and the USA. Grey shading indicates the States (CA = California, DE = Delaware, GA = Georgia, ME = Maine, MD = Maryland, MI = Michigan, NJ = New Jersey, NY = New York, NC = North Carolina, PA = Pennsylvania, OR = Oregon, WA = Washington) and Province (ON = Ontario) where sampling took place in the present study. Blue circles represent parasitoids obtained from sentinel baits and as by-catch in *Drosophila suzukii* traps, green circles represent parasitoids reared from ripe fruit collections and red circles show the absence of parasitoids from fruit collections. Note that adventive *L. japonica* was already previously reported from British Columbia (BC), Canada (Abram et al. 2020) and Washington, USA (Beers et al. 2022) and these previous finding were not included in the present study.

Only three additional species of parasitoids were obtained in the fruit collections: *A.* cf. *rufescens* emerged from fruit collections in Delaware and Washington (representing 0.2% of the 2884 parasitoids collected from all sites), *Pachycrepoideus vindemiae* Rondani (Hymenoptera, Pteromalidae) emerged from fruit collections in Oregon (representing 0.5% of the 2884 parasitoids collected) and *Trichopria drosophilae* Perkins (Hymenoptera, Diapriidae) emerged from a single collection in California, representing 1.2% of the 2884 parasitoids collected (Table 2; Suppl. material 3). It is likely that the two pupal parasitoids, *P. vindemiae* and *T. drosophilae*, were present in other locations as well; however, this study was focused on figitid larval parasitoids.

Drosophila suzukii-baited sentinels

In total, 120 parasitoid specimens were obtained from baited sentinels deployed in Oregon, New Jersey, North Carolina, Maine and Georgia. Using a combination of DNA barcoding and multiplex PCR, 117 specimens were identified at the species

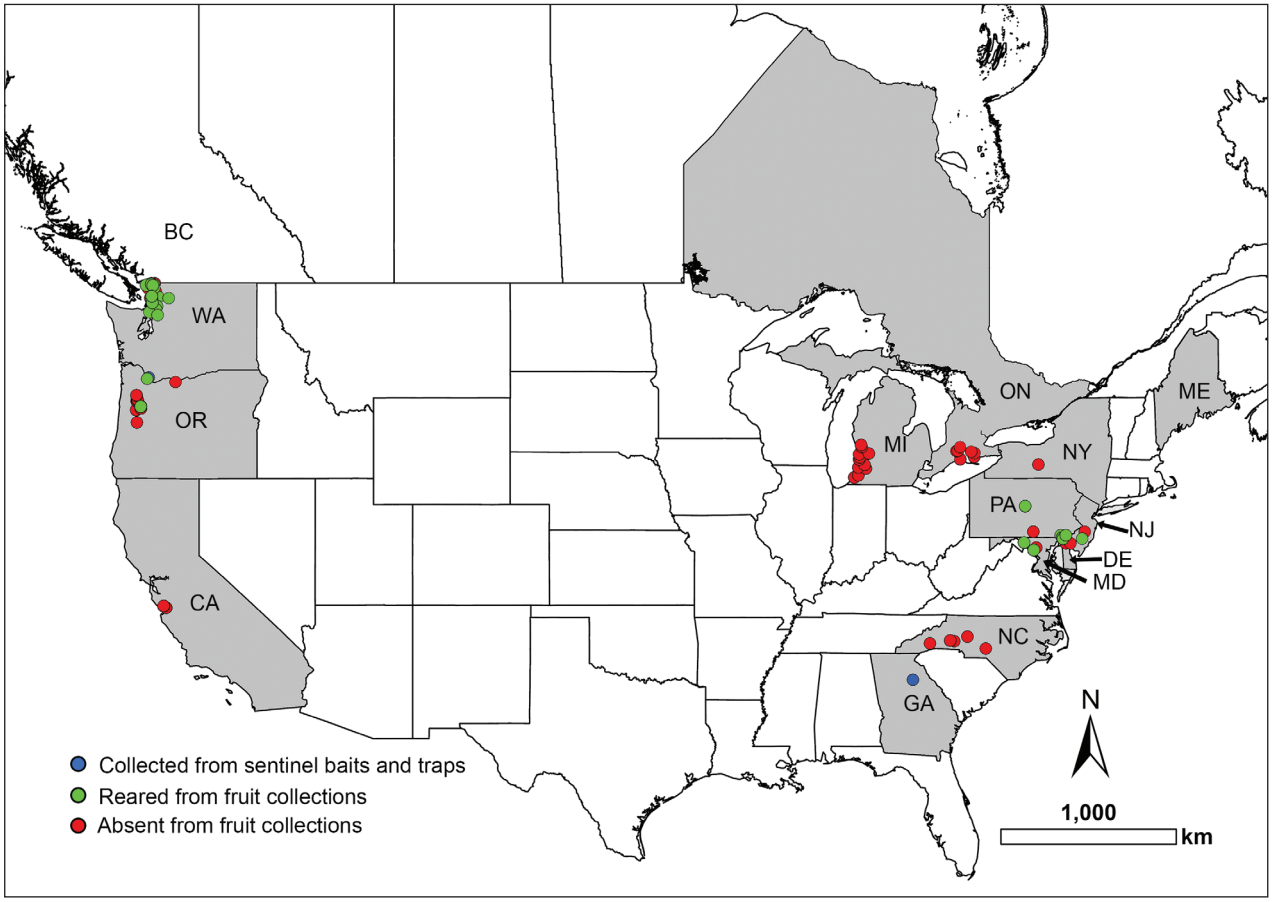

Figure 2. Distribution of *Ganaspis* cf. *brasiliensis* G1 detections after intended releases in 2022 in the USA. Grey shading indicates the States (CA = California, DE = Delaware, GA = Georgia, ME = Maine, MD = Maryland, MI = Michigan, NJ = New Jersey, NY = New York, NC = North Carolina, PA = Pennsylvania, OR = Oregon, WA = Washington) and Province (ON = Ontario) where sampling took place in the present study. Blue circles represent parasitoids obtained from sentinel baits and as by-catch in *Drosophila suzukii* traps, green circles represent parasitoids reared from ripe fruit collections and red circles show the absence of parasitoids from fruit collections. Note that adventive *G.* cf. *brasiliensis* was already previously reported from British Columbia, Canada (Abram et al. 2020) and Washington, USA (Beers et al. 2022) and these previous finding were not included in the present study.

level; only three samples from North Carolina failed to amplify or sequence, which may have been due to delayed preservation following collection (Suppl. material 1). Two species were detected: *L. japonica* (98%) and *G.* cf. *brasiliensis* G1 (2%). *Leptopilina japonica* was found in all four States where parasitoids were identified, whereas *G.* cf. *brasiliensis* G1 was only detected in New Jersey (one individual, collected 03 August 2022) and Georgia (one individual, collected 31 October 2022) (Table 3, Fig. 2). These *G.* cf. *brasiliensis* G1 detections occurred after the intended biological control releases of this parasitoid species took place in New Jersey and Georgia in summer 2022.

Opportunistic sampling of by-catch from other trap types

Seventy-one Figitidae parasitoid specimens were retrieved as by-catch in traps from Ontario, Washington and North Carolina. As these are non-specific traps attracting a variety of Drosophilidae and their parasitoids, a more diverse parasitoid

community, represented by five species, was captured. Collectively, the majority were *L. japonica* (72%), followed by *L. heterotoma* (9.9%), *L. leipsi* (7%), *L. maia* (2.8%) and *G.* cf. *brasiliensis* G1 (2.8%); the remaining specimens (5.5%) were unidentified using molecular techniques (DNA barcoding and multiplex PCR), possibly due to DNA degradation caused by prolonged immersion in drowning fluids (e.g. vinegar) that can degrade the quality of DNA. The parasitoid species composition from each State and Province sampled is shown in Table 3. All three jurisdictions were positive for the presence of adventive *L. japonica*, whereas *G.* cf. *brasiliensis* G1 was only detected in samples from Washington, which is consistent with the other types of collections.

Molecular identification tools for parasitoids of *Drosophila suzukii*

DNA barcoding of specimens

A total of 653 field-collected parasitoid adults (obtained through direct sampling of fruits, sentinels and other traps) were obtained for DNA barcoding, of which 494 produced complete DNA barcode sequences that permitted identification (Fig. 3). Approximately 24% of the samples failed to produce high-quality DNA

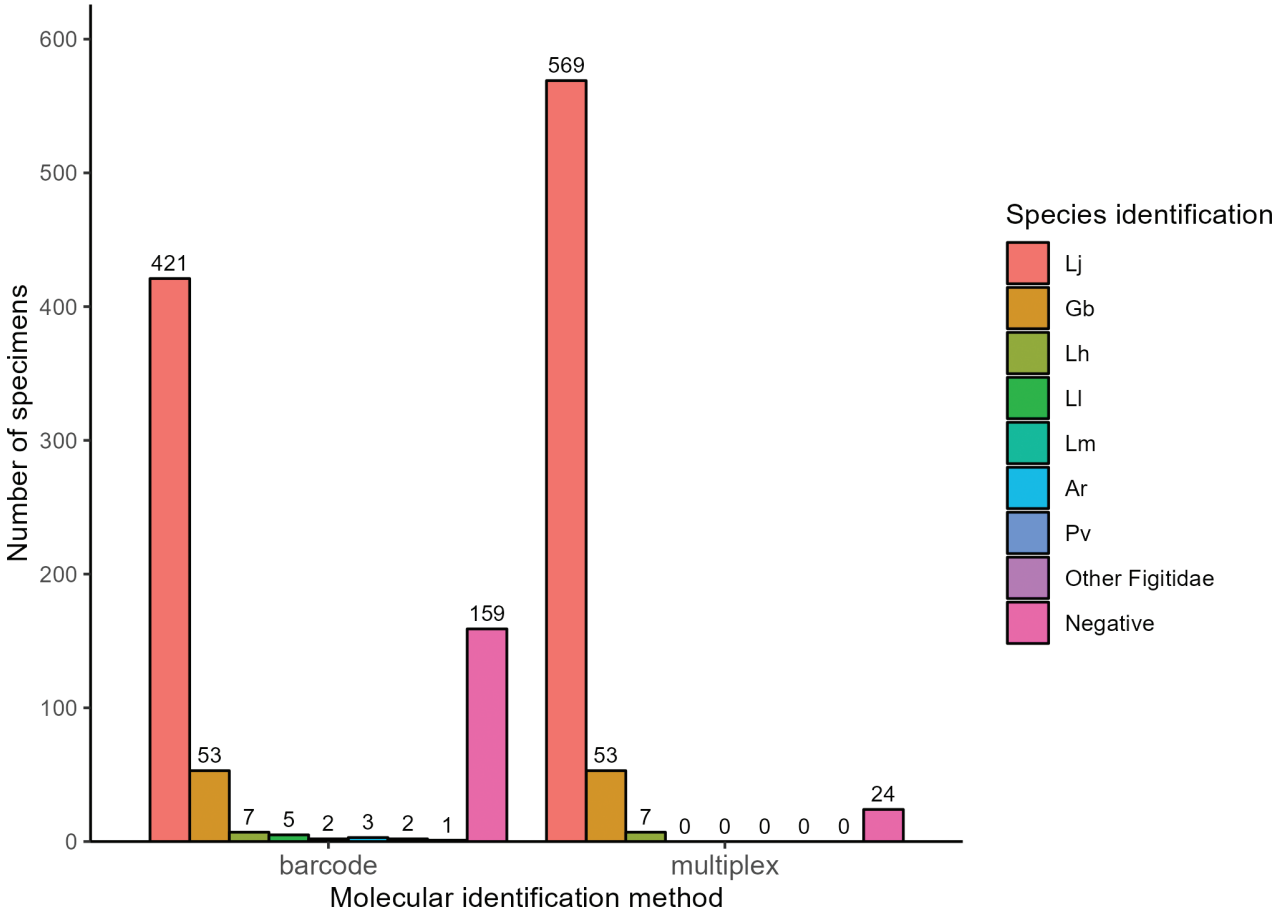

Figure 3. Identification of the same set of field-collected parasitoids (n = 653) using DNA barcoding and a multiplex PCR assay for *L. japonica* (Lj), *L. heterotoma* (Lh) and *G.* cf. *brasiliensis* G1 (Gb). Ar = *Asobara* cf. *rufescens*, Ll = *Leptopilina leipsi*, Lm = *Leptopilina maia*, Pv = *Pachycrepoideus vindemiae*. Negative refers to samples which failed to yield a DNA barcode or a PCR product.

sequences, likely due to DNA degradation from delayed preservation or improper storage of specimens following emergence. Nonetheless, 76% of the samples were identified using DNA barcoding. The vast majority of parasitoids collected were the Asian parasitoids, *L. japonica* (65%) and *G.* cf. *brasiliensis* G1 (8%); the remaining specimens were infrequently collected and occupied 0.30–1.1% of the species composition (Fig. 3).

Of the identified specimens obtained from USDA-ARS BIIRU laboratory colonies, 53 of the 64 that were used as references [15 *G.* cf. *brasiliensis* G1, 10 *G.* cf. *brasiliensis* G3, 10 *L. japonica* (originating from China), 9 *L. japonica* (originating from South Korea) and 20 *A. japonica* (originating from South Korea)] yielded DNA barcode compliant sequences (GenBank Accession numbers: OR974845–OR974897). None of the *L. japonica* from China produced viable barcode sequences (five contained stop codons and the other five produced poor quality sequences). In contrast, all *L. japonica* from South Korea yielded DNA barcodes. Only one *G.* cf. *brasiliensis* G3 failed to amplify and sequence. The failure to sequence from these few specimens is likely due to delayed preservation of wasps prior to DNA extraction. This was the case for specimens of *L. japonica* from China, where wasps were not freshly killed, but were stored dry for some time before preservation in 95% ethanol.

Development of multiplex PCR primers

When used in multiplex, the primers retained their specificity and amplified the correct fragment size for the intended target species when challenged with DNA from *G.* cf. *brasiliensis* G1, *G.* cf. *brasiliensis* G3, *L. japonica* (China), *L. japonica* (South Korea), *L. heterotoma*, *A.* cf. *rufescens*, *A. japonica*, *P. vindemiae*, *L. maia* and *L. leipsi* (Fig. 4). The *G.* cf. *brasiliensis* G1 primers only amplified the G1 strain of *G.* cf. *brasiliensis* and they did not amplify the G3 strain, which is consistent with how the primers were designed (i.e. to amplify specifically G1 and not G3). The *L. japonica* primers amplified all specimens of *L. japonica* from China and South Korea, even though specimens of *L. japonica* from China did not produce a viable barcode. This suggests that the specimens that failed to produce a barcode were incorrectly preserved, resulting in degraded DNA, which is also supported by a fainter PCR fragment visualised for these specimens (Fig. 4). *Pachycrepoideus vindemiae*, *L. leipsi*, *L. maia*, *A. japonica* and *A.* cf. *rufescens* were not amplified in the multiplex PCR.

When applied to the 653 field-collected specimens, all identifications were consistent with the barcoding results and the majority of parasitoids were identified as *L. japonica* (87%) and *G.* cf. *brasiliensis* G1 (8%), with a minor contribution from *L. heterotoma* (1%) (Fig. 3; Suppl. material 1). Only 25 samples (4%) failed to produce a PCR product using the multiplex PCR protocol, thereby allowing identification of 96% of the samples (Fig. 3). Of the specimens that yielded a negative PCR result, eleven also failed to produce a DNA barcode, indicating that the DNA was of insufficient quality for amplification. The remaining 14 samples that yielded a negative PCR result were identified by DNA barcoding as *A.* cf. *rufescens* (3), *L. leipsi* (5), *L. maia* (2), *P. vindemiae* (2) and unidentified Figitidae (2); thus, these samples were negative because they are not amongst the species targeted by the multiplex PCR assay.

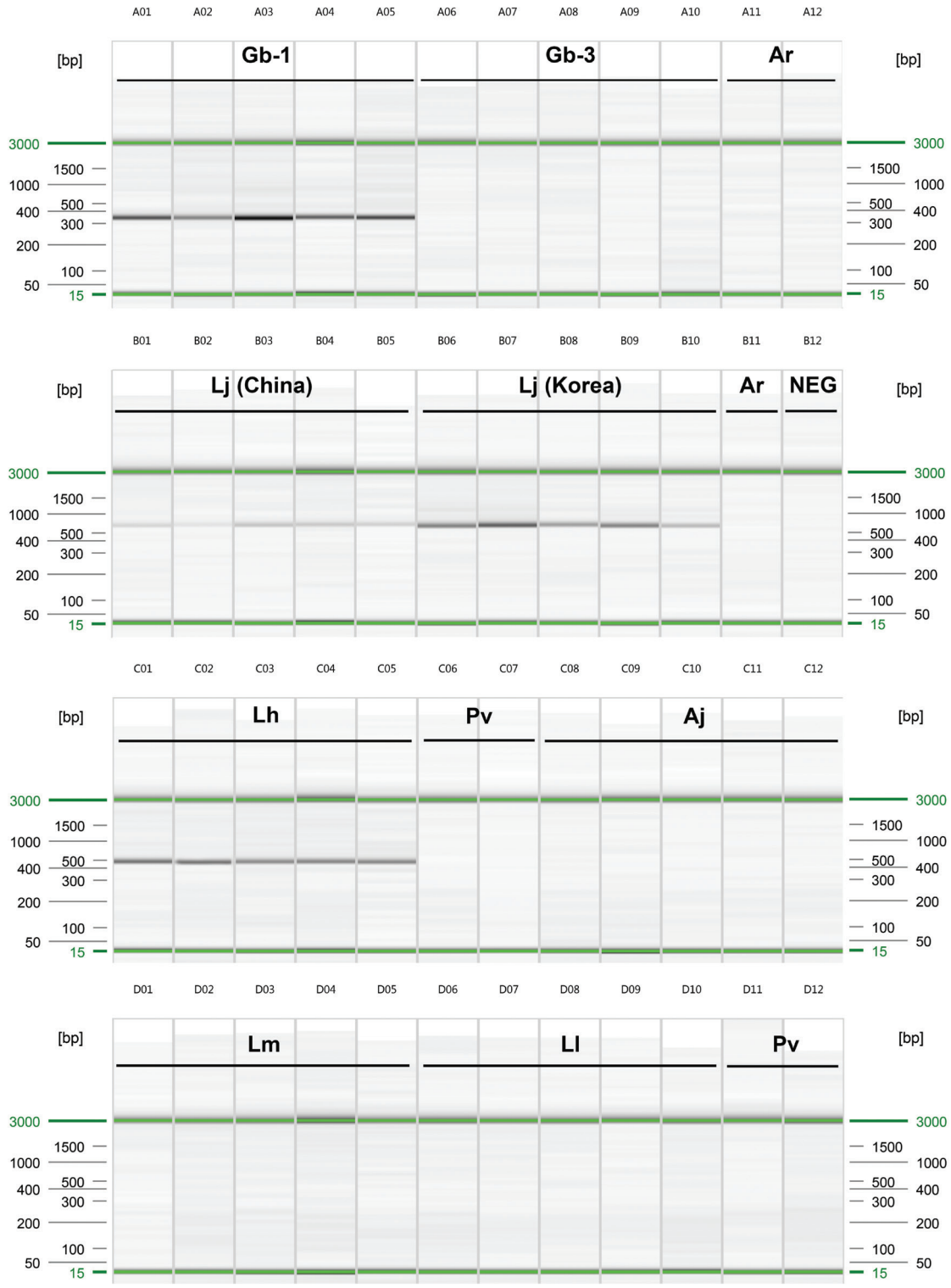

Figure 4. Multiplex PCR assay challenged with DNA from *Ganaspis* cf. *brasiliensis* G1 (Gb-1: A01–A05) and G3 (Gb-3: A06–A10), *Asobara* cf. *rufescens* (Ar: A11, A12, B11), *Leptopilina japonica* (China: B01–B05; South Korea: B06–B10), *Leptopilina heterotoma* (Lh: C01–C05), *Pachycrepoideus vindemiae* (Pv: C06, C07, D11, D12), *Asobara japonica* (Aj: C08–C12), *Leptopilina maia* (Lm: D01–D05), *Leptopilina leipsi* (Ll: D06–D10) and a negative control (NEG) with no DNA. Alignment markers (in green) are shown at 15 bp and 3000 bp for all samples and positive PCR results are indicated by fragment sizes of 332 bp, 639 bp and 473 bp for Gb-1, Lj and Lh (respectively). Absence of a fragment between the 15 bp and 3000 bp alignment markers indicates a negative PCR result.

Discussion

The geographic range of *L. japonica* was historically restricted to Asia and although this species was not being considered as a candidate agent for intentional biological control introductions, this study reveals that it is now present throughout a large part of North America and appears to be the dominant parasitoid associated with invasive *D. suzukii* at this point in time. This represents one of few documented cases of what appears to be a rapid (< 10 years) continent-wide spread of an unintentionally introduced parasitoid wasp, with the most similar example being the ongoing spread of unintentionally introduced egg parasitoids of *Halyomorpha halys* Stål (Hemiptera, Pentatomidae) in North America and Europe (Talamas et al. 2015; Stahl et al. 2019a).

This study reports a considerable North American range expansion for *L. japonica*, with new detections of *L. japonica* in 10 USA States (Delaware, Georgia, Maine, Maryland, Michigan, New Jersey, New York, North Carolina, Oregon and Pennsylvania) and one Canadian Province (Ontario). We also confirmed its establishment in Washington State, where it had already been reported (Beers et al. 2022) following its initial detection in adjacent British Columbia, Canada in 2016 (Abram et al. 2020). Only one USA State surveyed, California, did not detect any *L. japonica*; however, 2022 collections in this State were focused on a single location. Although the timeline of *L. japonica*'s unintentional introduction and spread in North America is somewhat uncertain, it seems most likely that it has occurred relatively recently, i.e. since the widespread establishment of *D. suzukii* (~ 2011). This is supported by both published (Thistlewood et al. 2013; Miller et al. 2015; Lue et al. 2016; Wang et al. 2016; Huang et al. 2023) and unpublished survey results (see Suppl. material 4) from several locations in North America that took place between 2011 and 2021 that did not recover any evidence of the presence of *L. japonica*. The only prior reports of *L. japonica* were recent; in the Pacific Northwest by Abram et al. (2020) and Beers et al. (2022) and, in Oregon, where parasitoids recovered from an extensive fruit collection in September 2021 were later identified as *L. japonica* (Suppl. material 4). Similarly, in Europe, where there has been a considerable amount of field research on parasitoids of *Drosophila* spp. (Rossi Stacconi et al. 2013; Englert and Herz 2016; Mazzetto et al. 2016; Knoll et al. 2017; Kremmer et al. 2017; Shaw et al. 2023), it seems that *L. japonica* has only arrived since the introduction of *D. suzukii* (Puppato et al. 2020; Fellin et al. 2023; Martin et al. 2023). These observations support the "receptive bridgehead hypothesis" (Weber et al. 2021), in which unintentional introductions of biological control agents to new areas may be more likely following invasions of those areas by suitable hosts from their native range. As *L. japonica* has a host range that includes several native and cosmopolitan species of Drosophilidae other than *D. suzukii*, including the abundant and widespread *Drosophila melanogaster* Meigen (Girod et al. 2018b; Giorgini et al. 2019; Daane et al. 2021; Fellin et al. 2023), the *L. japonica* "invasion" has the potential to have both positive economic and environmental impacts [via suppression of *D. suzukii* populations (and/or other pestiferous *Drosophila* spp.)] and potential ecological harm (direct and indirect effects of attacking native and cosmopolitan Drosophilidae) that should be evaluated in the coming years.

Unintentionally introduced populations of *G.* cf. *brasiliensis* G1 (the more specialised parasitoid of *D. suzukii* recently approved for intentional releases in North

America and Europe) do not appear to be nearly as widespread as *L. japonica*. However, it is important to note that this species was only recently released and is in the early stages of establishment in the locations surveyed in the present study. In Washington, populations of *G.* cf. *brasiliensis* were detected prior to intentional releases and the present study suggests additional spread of adventive populations to new locations within the state. However, the detection of *G.* cf. *brasiliensis* in the additional 6 US States (Delaware, Georgia, Maryland, New Jersey, Pennsylvania and Oregon) reported here only occurred in 2022, following intentional releases. There are records of *G.* cf. *brasiliensis* from tropical and sub-tropical regions of the Palearctic (Uganda), Nearctic (Mexico), Neotropical (Panama, Brazil) and Oceania (Hawai'i) (Buffington and Forshage 2016), but these represent strains of this species other than G1 that are likely to be described as distinct species (M. Buffington, in prep.). Given the absence of previous records of *G.* cf. *brasiliensis* outside of the Pacific Northwest and the very low abundance of *G.* cf. *brasiliensis* G1 relative to *L. japonica* outside of this area, we conclude that most of the *G.* cf. *brasiliensis* G1 identified in the present study represent detections of this parasitoid resulting from the intentional releases (and not adventive populations). Although it is possible that the *G.* cf. *brasiliensis* G1 detections in Oregon were from adventive populations (given the proximity to Washington where populations were present before intentional releases took place; Beers et al. (2022)), extensive releases of *G.* cf. *brasiliensis* G1 took place in Oregon in 2022, with the only detections occurring after release. As the DNA barcodes for both adventive and intentionally-released populations are identical (Abram et al. 2020; Lue et al. 2021), the methods used in the present study cannot distinguish between post-release detections and the spread of already-established adventive populations. Other methods and continued surveys will be needed to tease apart the contribution of released and adventive populations of *G.* cf. *brasiliensis* G1 to its range expansion in North America in the coming years.

The present study also yielded new information on the distribution of native and cosmopolitan parasitoids associated with Drosophilidae. Native and cosmopolitan *Leptopilina* species (*L. maia*, *L. leipsi* and *L. heterotoma*) were only found in the vinegar and Scentry traps and not in fruit collections or sentinel baits, as they are not known to parasitise *D. suzukii* in the field and these traps are not specific to *D. suzukii*. *Leptopilina maia* was identified from trap catches in North Carolina (USA) and Ontario (Canada). The occurrence of *L. maia* in North Carolina is consistent with the distribution of this species in eastern North America (Lue et al. 2016); however, this is the first time this species has been reported in Canada. Similarly, although known from New Hampshire, Illinois, Maryland and Virginia (Lue et al. 2016), this is the first report of *L. leipsi* in Canada. Both species were recently described by Lue et al. (2016) and the present study provides updated distribution records. The records of the cosmopolitan species *L. heterotoma* from Ontario are also the first official records of this species in Canada, although re-inspection of historically collected specimens from British Columbia (reported erroneously in Thistlewood et al. (2013) as "*Ganaspis* sp.") indicates that it has been present in Canada since at least 2011 (P.K. Abram and D.R. Gillespie, unpublished data). The amount of new information about figitid geographic distributions gathered by this single study highlights the paucity of data on this important group of parasitoids and the uncertainty associated with inferring recent spread and establishment from survey data on understudied species groups.

Although the routes of invasion have been reconstructed for the global movement and spread of *D. suzukii* (Fraimout et al. 2017), the pathways of adventive introduction of *L. japonica* and *G.* cf. *brasiliensis* G1 are currently unknown and it is not yet clear whether they represent a single introduction with subsequent spread of the same introduced population or whether multiple introduction events have occurred. Given the large geographic distance between the initial introductions in the Pacific Northwest and the more recently-discovered establishments in eastern North America, it is possible that more than one introduction event took place and/or that a bridgehead event occurred, in which one introduced population served as the source for rapid dispersal and establishment of additional populations in North America (Guillemaud et al. 2011). However, further population genetic analysis of native and introduced populations would be necessary to identify the source population(s) and to trace the potential routes of entry and spread. Although the present study generated DNA barcode sequences for adventive populations in North America, sequences from additional genes and populations from across the native (and recently invaded) range could clarify source populations and infer pathways of entry. Further, additional genetic approaches may provide a higher resolution of population differences. For example, when COI barcode sequences were not adequate to detect population-level differences, ddRADseq was used to clarify relationships amongst adventive populations of *Trissolcus japonicus* Ashmead (Hymenoptera, Scelionidae), a parasitoid of *H. halys* (Abram et al. 2023) and between different populations of *D. suzukii* in Canada (Nelson et al. 2023).

Detection of introduced and adventive parasitoids in an invasive pest population is critical to document the establishment and spread of biological control agents, assess their effectiveness and evaluate their potential for long-term suppression of an invasive pest species (Gariepy et al. 2007; Furlong 2015; Lue et al. 2022). To do this, large numbers of samples may need to be collected and processed to detect parasitism and parasitoid species composition. The availability of a molecular diagnostic tool can facilitate the delivery of these data and provide information for subsequent pest management strategies, including continued release and redistribution of biological control agents (Gariepy et al. 2007, 2008). Monitoring efforts to detect new adventive populations and to assess the post-release establishment following intended releases can be facilitated using the multiplex PCR approach described here. Our method provides a rapid, accurate and cost-effective option for screening field-collected samples that does not require the additional expense of DNA sequencing. It also facilitates conclusive identification of species that would otherwise be difficult to separate, based solely on morphological features (e.g. *L. japonica* versus *L. heterotoma*; G1 versus G3 *G.* cf. *brasiliensis*). This is an important consideration when screening large numbers of samples for these species, as the cost of identifying a sample through bidirectional sequencing is approximately tenfold more than identification using the multiplex PCR protocol described here. Furthermore, the species-specific primers more reliably provided species identifications than sequencing the entire barcode region (Fig. 3), likely because they generate a shorter fragment of DNA which can improve identification of samples that fail to sequence due to DNA degradation (Strutzenberger et al. 2012; Hebert et al. 2013; Mitchell 2015). This multiplex PCR may also be useful to detect ecological interactions that are difficult to address using conventional techniques, including potential competitive interactions between parasitoids, detection of host-parasitoid associations and evaluation of the host range in the area of introduction (e.g. Gariepy

and Messing (2012); Gariepy et al. (2019); Lomeli-Flores et al. (2019); Stahl et al. (2019b); Hepler et al. (2020)). It is important to note that, in the current context of screening figitid parasitoids that emerge from *D. suzukii* in ripe and sentinel fruit and species attracted to baited traps, the multiplex PCR protocol is effective and accurate. However, it may be prudent to screen additional verified species of Figitidae with this multiplex PCR protocol to ensure specificity is retained if this protocol is used in a different context with a more diverse parasitoid community (for example, figitids associated with other drosophilid species or for use in other countries with a different parasitoid complex associated with *D. suzukii*).

Conclusions

Our findings raise several questions relevant to interpreting the recent trend of unintentionally introduced populations of parasitoid wasps being detected during the course of biological control programmes. For example, is the apparently more widespread establishment of *L. japonica* compared to *G.* cf. *brasiliensis* G1 due to the fact that it has been established in these locations for a longer period of time? Or is it due to its broader host range and/or a broader climatic tolerance? If so, does that mean that parasitoids that are less likely to be approved for intentional biological control releases are more likely to be unintentionally introduced and become established? If the routes of introduction needed to spread *L. japonica* to multiple areas of the world are present and this parasitoid can attack Drosophilidae species other than *D. suzukii*, why did it seemingly not establish outside of Asia before the *D. suzukii* invasion? If a broader host range makes a parasitoid species more likely to be unintentionally introduced to new areas, why is *A. japonica*, a very common parasitoid of *D. suzukii* in some areas of its native range and which attacks a wide range of Drosophilidae species (Daane et al. 2021), not yet present in North America or Europe? The relative contributions of several factors to unintentional parasitoid establishment (e.g. parasitoid host range; parasitoid prevalence in source areas; climatic tolerance; patterns of global trade driving pest and parasitoid propagule pressure; host abundance in space and time) should be the focus of future work. Indeed, our study contributes to the increasing recognition that parasitoid wasps are part of the rising number of non-native insect introductions and the potential for them to have widespread positive, negative and mixed ecological impacts is considerable. However, the factors that contribute to their global spread and what this means for biological control of invasive species and risks to native ecosystems, deserve more attention.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Conceptualization: BH, PKA, JCL, KAH, PF, KD, EB, CRS, HKL, TDG, HB, XW, KMD, MLB, RI, VMW. Data curation: KH, HKL, SVT, TDG, BJ, BH, AAS, CA, JKW, AB. Formal analysis: JCL, TDG, XW, PKA. Funding acquisition: KAH, KMD, RI, TDG. Investigation: GB, DB, TDG, EB, JCL, BH, KMD, XW, GL, RI, PF, AS, KH, BJ, HB, CRS, PP, AG, AL, JB, KD. Methodology: XW, GL, JCL, JKW, CA, KRP, MLB, AS, TDG, VMW, SVT, HB, JMMM, DB, HKL, KR, CRS, PP, CJ, KD, SN, PS, DB, JMR, AB. Resources: VMW, TDG, BH, CRS. Validation: TDG. Visualization: XW, TDG, PKA. Writing - original draft: XW, PKA, TDG. Writing - review and editing: JCL, RI, EB, AAS, PF, MLB, KMD, KAH, KH, VMW, BJ, CA, SVT, BH, TDG, PS, HKL, PKA, XW, DB, GL, JMR, CRS, DB.

Author ORCIDs

Tara D. Gariepy https://orcid.org/0000-0003-2533-3023
Dylan Beal https://orcid.org/0000-0001-5965-9043
Elizabeth Beers https://orcid.org/0000-0003-4917-9518
Matthew Buffington https://orcid.org/0000-0003-1900-3861
Kathleen Demchak https://orcid.org/0009-0004-2143-1616
Alexandra Gillett https://orcid.org/0000-0003-4477-4270
Rufus Isaacs https://orcid.org/0000-0001-7523-4643
Hannah K. Levensen https://orcid.org/0000-0002-1667-0127
Justin M. Renkema https://orcid.org/0000-0003-2165-660X
Cesar Rodriguez-Saona https://orcid.org/0000-0002-1030-2753

Subin Neupane https://orcid.org/0000-0000-0000-0000
Steven Van Timmeren https://orcid.org/0000-0003-2555-4950
Julianna K. Wilson https://orcid.org/0000-0003-0807-5421
Xingeng Wang https://orcid.org/0000-0002-8825-2266

Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Collection information and molecular identification of parasitoids reared from field-collected ripe fruit (GPS coordinates for the general site vicinity were used to preserve privacy of landowners)

Authors: ara D. Gariepy, Paul K. Abram, Chris Adams, Dylan Beal, Elizabeth Beers, Jonathan Beetle, David Biddinger, Gabrielle Brind'Amour, Allison Bruin, Matthew Buffington, Hannah Burrack, Kent M. Daane, Kathleen Demchak, Phillip Fanning, Alexandra Gillett, Kelly Hamby, Kim Hoelmer, Brian Hogg, Rufus Isaacs, Ben Johnson, Jana C. Lee, Hannah K. Levensen, Greg Loeb, Angela Lovero, Joshua M. Milnes, Kyoo R. Park, Patricia Prade, Karly Regan, Justin M. Renkema, Cesar Rodriguez-Saona, Subin Neupane, Cera Jones, Ashfaq Sial, Peter Smythman, Amanda Stout, Steven Van Timmeren, Vaughn M. Walton, Julianna K. Wilson, Xingeng Wang

Data type: xlsx

Link: https://doi.org/10.3897/neobiota.93.121219.suppl1

Supplementary material 2

DNA barcoding of specimens

Authors: ara D. Gariepy, Paul K. Abram, Chris Adams, Dylan Beal, Elizabeth Beers, Jonathan Beetle, David Biddinger, Gabrielle Brind'Amour, Allison Bruin, Matthew Buffington, Hannah Burrack, Kent M. Daane, Kathleen Demchak, Phillip Fanning, Alexandra Gillett, Kelly Hamby, Kim Hoelmer, Brian Hogg, Rufus Isaacs, Ben Johnson, Jana C. Lee, Hannah K. Levensen, Greg Loeb, Angela Lovero, Joshua M. Milnes, Kyoo R. Park, Patricia Prade, Karly Regan, Justin M. Renkema, Cesar Rodriguez-Saona, Subin Neupane, Cera Jones, Ashfaq Sial, Peter Smythman, Amanda Stout, Steven Van Timmeren, Vaughn M. Walton, Julianna K. Wilson, Xingeng Wang

Data type: docx

Link: https://doi.org/10.3897/neobiota.93.121219.suppl2

Supplementary material 3

All fruit samplings (GPS coordinates for the general site vicinity were used to preserve privacy of landowners)

Authors: ara D. Gariepy, Paul K. Abram, Chris Adams, Dylan Beal, Elizabeth Beers, Jonathan Beetle, David Biddinger, Gabrielle Brind'Amour, Allison Bruin, Matthew Buffington, Hannah Burrack, Kent M. Daane, Kathleen Demchak, Phillip Fanning, Alexandra Gillett, Kelly Hamby, Kim Hoelmer, Brian Hogg, Rufus Isaacs, Ben Johnson, Jana C. Lee, Hannah K. Levensen, Greg Loeb, Angela Lovero, Joshua M. Milnes, Kyoo R. Park, Patricia Prade, Karly Regan, Justin M. Renkema, Cesar Rodriguez-Saona, Subin Neupane, Cera Jones, Ashfaq Sial, Peter Smythman, Amanda Stout, Steven Van Timmeren, Vaughn M. Walton, Julianna K. Wilson, Xingeng Wang

Data type: xlsx

Link: https://doi.org/10.3897/neobiota.93.121219.suppl3

Supplementary material 4

Collection data

Authors: ara D. Gariepy, Paul K. Abram, Chris Adams, Dylan Beal, Elizabeth Beers, Jonathan Beetle, David Biddinger, Gabrielle Brind'Amour, Allison Bruin, Matthew Buffington, Hannah Burrack, Kent M. Daane, Kathleen Demchak, Phillip Fanning, Alexandra Gillett, Kelly Hamby, Kim Hoelmer, Brian Hogg, Rufus Isaacs, Ben Johnson, Jana C. Lee, Hannah K. Levensen, Greg Loeb, Angela Lovero, Joshua M. Milnes, Kyoo R. Park, Patricia Prade, Karly Regan, Justin M. Renkema, Cesar Rodriguez-Saona, Subin Neupane, Cera Jones, Ashfaq Sial, Peter Smythman, Amanda Stout, Steven Van Timmeren, Vaughn M. Walton, Julianna K. Wilson, Xingeng Wang

Data type: xlsx

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