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ORIGINAL ARTICLE

Assessing potential hybridization between a hypothetical gene drive-modified *Drosophila suzukii* and nontarget *Drosophila* species

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Abstract

Genetically engineered gene drives (geGD) are potentially powerful tools for suppressing or even eradicating populations of pest insects. Before living geGD insects can be released into the environment, they must pass an environmental risk assessment to ensure that their release will not cause unacceptable harm to non-targeted entities of the environment. A key research question concerns the likelihood that nontarget species will acquire the functional GD elements; such acquisition could lead to reduced abundance or loss of those species and to a disruption of the ecosystem services they provide. The main route for gene flow is through hybridization between the geGD insect strain and closely related species that co-occur in the area of release and its expected dispersal. Using the invasive spotted-wing drosophila, *Drosophila suzukii*, as a case study, we provide a generally applicable strategy on how a combination of interspecific hybridization experiments, behavioral observations, and molecular genetic analyses can be used to assess the potential for hybridization.

KEYWORDS
environmental risk assessment, invasive species, nontarget effects, pest control, spotted-wing drosophila

1 | INTRODUCTION

The advent of molecular and synthetic biology, including the development of CRISPR/Cas9 gene editing technology, has enabled scientists to engineer gene drive (GD) elements into insects. These elements can promote biased inheritance of genes by the next generation via mating, driving genes of interest through a target population even if they impose fitness costs to the carrier (Esvelt et al., 2014; L. S. Alphey et al., 2020). Gene drives are known to occur naturally in a number of organisms (Burt and Trivers, 2006). Depending on the trait(s) conferred to the genetically engineered (geGD) insect, engineered GDs can lead to population suppression (potentially even elimination) or modification. Thus, engineered GDs are currently being explored as tools to control or replace populations of harmful species (see Box 1 for terminology), especially invasive species and disease vectors such as mosquitoes, or to rescue endangered species (Champer et al., 2016; N. Alphey and Bonsall, 2018; James et al., 2018; Scott et al., 2018; Rode et al., 2019; Raynolds, 2021). As for any other genetic control method, the GD approach requires the release of genetically engineered insects into the environment (Teem et al., 2020).

A number of engineered GD systems have been developed (L. S. Alphey et al., 2020; Devos et al., 2021). They can be categorized depending on the threshold density at which they must be released into a population in order for the GD to be effective. So-called low-threshold drives can spread even when a small number of geGD insects are released, while high-threshold drives require the presence of a substantial number of geGD individuals in a population. GDs can also be designed to be self-sustaining (i.e., to persist indefinitely) or self-limiting (i.e., to be restricted in spread and/or persistence). As a consequence, one can expect the effect of

self-limiting, high-threshold GDs to be much more localized than that of low-threshold, self-sustaining ones.

Although no insects with engineered GDs have to this date been released and tested in the field, many are being studied and developed under laboratory conditions. For some strains, population suppression has already been demonstrated in cage experiments. Examples for suppression geGDs include the exploitation of a *doublesex* gene that leads to complete infertility in homozygous engineered females of the mosquito *Anopheles coluzzii* (Diptera: Culicidae) (Kyrou et al., 2018; Hammond et al., 2021) and a synthetic *Medea* (Maternal Effect Dominant Embryonic Arrest) GD system based on a toxin that is expressed in the modified mothers coupled with an embryonic antidote in the spotted-wing drosophila, *Drosophila suzukii* (Diptera: Drosophiliidae) (Buchman et al., 2018). Modification GDs have been successfully introduced into, for example, malaria vectors of the genus *Anopheles* with the aim of impairing the ability of females to transmit the *Plasmodium* parasite (Gantz et al., 2015; Adolfi et al., 2020; Carballar-Lejarazú et al., 2020).

Concerns have been raised that the release of geGD insects could lead to undesired effects on the environment including reductions in biodiversity and ecosystem services (Gould, 2008; NASEM, 2016; Hayes et al., 2018; Romeis et al., 2020; Devos et al., 2022; Kokotovich et al., 2022). Because they are genetically engineered (GE) organisms, geGD insects are subject to regulatory approval under all jurisdictions. Consequently, an environmental risk assessment (ERA) must be conducted before their intentional release (Devos et al., 2021; Devos et al., 2022; Devos et al., 2022b; Tonui et al., 2022).

The first step in any ERA is problem formulation (Raybould, 2006; Devos et al., 2019; Teem et al., 2019). In this step, relevant protection goals are identified and plausible pathways (i.e., causal chains of events) through which the release of the geGD insects could harm those protection goals are identified. This allows researchers to formulate risk hypotheses for the consecutive events in the "pathways-to-harm" and to identify the data/information required to subsequently test those hypotheses with the goal of characterizing the risk (Connolly, Mumford et al., 2022). Plausible pathways-to-harm have already been determined for geGD insects using examples including *Anopheles gambiae* (Diptera: Culicidae) (the GD is designed to reduce malaria transmission in Africa) (Teem et al., 2019; Connolly et al., 2021) and the invasive agricultural pest *D. suzukii* (the GD is designed to reduce the pest's population in the invaded areas and consequently the damage caused) (Romeis et al., 2020).

A key question asked in an ERA is whether the potential acquisition of functional GD elements by nontarget species could cause harm to the environment (Gould, 2008; Rode et al., 2019; Lalyer et al., 2021). Depending on the engineered GD, the transfer of the GD element to populations of taxonomically closely related nontarget species through mating (vertical gene flow) or horizontal gene transfer to sexually incompatible species may lead to the suppression or extinction of a nontarget population or even species. The environmental risk related to gene flow has so far been explored conceptually (using a modeling approach) (Courtier-Orgogozo et al., 2020) and experimentally for disease-transmitting *Anopheles* spp. (e.g., Hanemaaijer et al., 2018; Bernardini et al., 2019). Although there is a small chance for gene flow to occur through horizontal gene transfer, the more likely route of exposure is through hybridization of the geGD organism with closely related species (NASEM, 2016). Successful hybridization between a geGD insect strain and taxonomically related species therefore represents a pivotal event in a number of pathways-to-harm affecting nontarget species, ecosystem services, and other valued components of the environment (Romeis et al., 2020; Connolly et al., 2021) (note that gene flow within a target species complex might be envisaged, Connolly, Romeis et al., 2022). Consequently, case-specific ERAs for the environmental release of a geGD insect must consider the potential of the organism to hybridize with nontarget species (NASEM, 2016; Tonui et al., 2022). Guidance on how to assess this potential, however, is lacking (Devos et al., 2022). We here investigate the hybridization potential using *D. suzukii* as a model case and examine which information is required to test the risk hypothesis that a particular geGD insect does not hybridize with nontarget species.

The spotted-wing drosophila, *D. suzukii*, originated in Eastern Asia and potentially has a very broad global distribution (dos Santos et al., 2017). It has been invasive in Europe and the Americas since 2008 (Asplen et al., 2015) and was recently reported from sub-Saharan Africa for the first time (Kwadha et al., 2021). Given the ability of this species to adapt to new environments, further spread is expected (Little et al., 2020). *Drosophila suzukii* has a serrated ovipositor and lays its eggs into undamaged ripening fruits and berries, causing major damage and revenue losses. Controlling this pest by conventional means (e.g., insecticides, antagonists, and behavioral manipulation) is very challenging, labor intensive, costly, and, in the case of insecticides, comes with environmental costs (Sarkar et al., 2020; Tait et al., 2021). Therefore, alternative control measures including genetic control approaches such as geGDs have been suggested (Schetelig et al., 2018; Romeis et al., 2020). In an agricultural context, *D. suzukii* is probably the most advanced geGD system in insects moving toward practical applications (Buchman et al., 2018; Schetelig et al., 2018; Scott et al., 2018). Its advanced status in terms of geGD application together with the fact that hybridization has been reported for closely related *Drosophila* species (Bock, 1984; Garrigan et al., 2012; Suvorov et al., 2022) makes *D. suzukii* a useful study case.

The case explored in the current research is based on a hypothetical *D. suzukii* strain that might be engineered with a GD for release in Central Europe to provide a large-scale suppression or even eradication of this invasive pest. We first identified the *Drosophila* species present in Europe, and then selected those that are taxonomically the most closely related to *D. suzukii* for hybridization experiments. These experiments were complemented with behavioral studies to

determine whether adults of the different species recognize each other as potential mating partners. In addition, molecular tools were developed and used to confirm the identities of adults used in hybridization experiments and to determine whether the progeny of presumed hybridization events was truly hybrids.

2 | METHODS AND MATERIALS

2.1 | Insect material

Species were selected for this study according to Bächli et al., 2004 and "The database on Taxonomy of Drosophilidae" (TaxoDros) (Bächli, 2022), which is regularly updated and contains the most comprehensive information on *Drosophila* spp. taxonomy and distribution.

Because hybridization is most likely to occur between phylogenetically closely related species, we have a priori identified the *Drosophila* species that occur in Europe and are most closely related to *D. suzukii* (Figure 1). The genus *Drosophila* is species-rich with a total of 1665 species registered in 2017 (O'Grady and DeSalle, 2018). *Drosophila suzukii* belongs to the *Drosophila melanogaster* species group (containing a total of 96 species) within the subgenus *Sophophora* (341 species) (O'Grady and Kidwell, 2002; Bächli, 2022). In Europe, only three species from the *D. melanogaster* species group in addition to *D. suzukii* are known to occur, i.e., *Drosophila ananassae*, *D. melanogaster*, and *Drosophila simulans* (Bächli et al., 2004). Therefore, those three species were selected for our experiments. The species groups are further divided into subgroups: one subgroup contains *D. suzukii*, a second subgroup contains *D. ananassae*, and a third subgroup contains *D. melanogaster* and *D. simulans* (Kopp, 2006) (Figure 1). The latter two species differ only in one large and a few small chromosome inversions and in only about 4%–8% in terms of DNA sequence (Davis et al., 1996). Hybridization between *D. melanogaster* and *D. simulans* has previously been reported, and rare cases of fertile hybrid females were observed for some strains but not for others (Lachaise et al., 1986; Davis et al., 1996; Sawamura, 2000). Therefore, this species pair served as a positive control for the hybridization experiments.

We also included *Drosophila biarmipes* as a fourth test species, as it belongs to the *D. suzukii* species subgroup (Kopp et al., 2019). This species, however, is only present in Southeast Asia and is partially sympatric with *D. suzukii* (Toda, 1991). Even though the phylogenetic relationships among the species within the *D. melanogaster* species group are not entirely resolved, molecular analyses suggest that the *D. melanogaster* and *D. ananassae* subgroups are each monophyletic, while the *D. suzukii* subgroup is polyphyletic (Kopp, 2006). However, *D. suzukii* and *D. biarmipes* belong to a monophyletic lineage within the *D. suzukii* subgroup (Kopp, 2006).

Drosophila ananassae, *D. biarmipes*, and *D. melanogaster* were obtained from the National Drosophila Species Stock Center, Cornell University (Ithaca, NY, USA) (stock numbers: 14024-0371.39, 14023-0361.06, and 14021-0231.149, respectively). A wild strain of *D. simulans* caught in Trentino, Italy, was provided by Valerio Mazzoni and Marco Valerio Rossi Stacconi from the Fondazione Edmund Mach (S. Michele all'Adige, Italy). *Drosophila suzukii* was collected in Zurich-Affoltern, Switzerland, and has been reared in our laboratory since 2013 (Knoll et al., 2017).

All *Drosophila* species were reared on a cornmeal diet adapted from the Cornell cornmeal recipe (Cornell University, 2022). Instead of the Tegosept and acid mixture, 3 g of Nipagin (4-hydroxybenzoesäure-methylester, Carl Roth GmbH, Karlsruhe, Germany) was added per 1 L of water. Approximately 50 three- to five-day-old fly pairs were kept in a 250-ml glass jar containing 50 ml of diet; a piece of kitchen towel was stuck into the diet as a substrate for the fly pupae. The jar was closed with a foam rubber plug. Flies were transferred to fresh jars after 2 days, and the procedure was repeated weekly with fresh flies. Two jars were prepared every week for each fly species.

To obtain virgin flies, all adult flies were removed from the rearing jars in the morning. After a maximum of 6 h, freshly emerged flies were collected by immobilizing them with CO_2; they were then sexed on a Flystuff Flypad (Genesee Scientific, El Cajon, CA, USA) with the aid of a stereomicroscope. Until they were used in the experiments, these flies

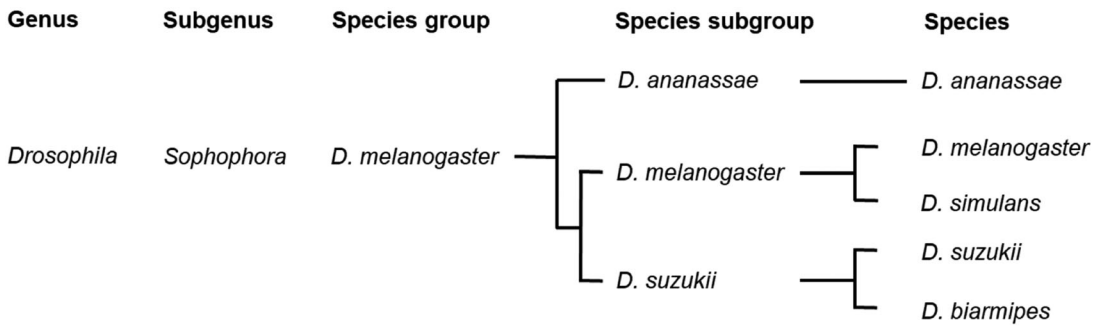

FIGURE 1 Phylogenetic relationships of the five *Drosophila* species used in the study (compiled according to Kopp, 2006; Kopp et al., 2019)

TABLE 1 Offspring resulting from the intra- and interspecific combinations of the five *Drosophila* species: *D. suzukii*, *D. ananassae*, *D. biarmipes*, *D. melanogaster*, and *D. simulans* (the age range [days] of mature female and male virgin flies is indicated)

	Females	***D. suzukii***	***D. ananassae***	***D. biarmipes***	***D. melanogaster***	***D. simulans***
Males	**Age (days)**	**2–4**	**4–7**	**2–5**	**2–5**	**3–5**
D. suzukii	2–5	46/46	0/42	0/42	0/42	0/42
D. ananassae	8–13	1/42	42/42			
D. biarmipes	2–5	1/42		42/42		
D. melanogaster	2–5	0/42			52/52	4/48
D. simulans	3–5	0/42			4/48	45/45

Note: The number of replicates with offspring per total number of replicates (e.g., 46/46) is indicated. Each replicate consisted of five females and five males. Empty cells indicate combinations that were not tested.

were stored by sex in groups of 25 in small plastic vials (62 mm height, 34 mm diameter) containing cornmeal diet at the bottom.

All flies were reared and experiments were conducted in climate chambers at 22°C, 70% relative humidity, and a 16:8 h light:dark photoperiod.

2.2 | Hybridization experiments

Fifteen intra- and interspecific combinations of the five *Drosophila* species were tested (Table 1). For each combination, five mature virgin females were paired with five mature virgin males in a plastic vial (67 mm height, 47 mm diameter) with approximately 20 ml of cornmeal diet and a piece of kitchen towel stuck into the diet. After 4 days (period 1), flies were transferred to a fresh vial and kept there for another 4 days (period 2). The vials were closed with foam rubber plugs. The age of mature flies differed among species and sex (see Table 1 for age of the mature flies); fly age was selected according to the age of reproductive maturity as determined in pre-trials (Figures S6–S15). The 8-day period was selected to maximize the chance for matings to occur.

The experiment was repeated seven to eight times, each with five to eight replicates per species combination, resulting in a total sample size of 42–52 per combination.

The vials were stored until the offspring emerged. Offspring were frozen in the vial and were subsequently counted and sexed. Potential hybrid offspring were stored at −20°C for molecular analyses.

2.3 | Mating behavior experiments

The mating behavior of male flies was observed in intraspecific combinations of all five *Drosophila* species and for interspecific reciprocal pairs of *D. suzukii* and each of the four other *Drosophila* species. One mature virgin male was combined with three mature virgin females (to provide a choice) in a small plastic vial (62 mm height, 34 mm diameter) that was closed with a foam rubber plug. The age of the flies used in this experiment varied to ensure maximal mating success. The age (females, males) of the different species was as follows: *D. ananassae* (3–10 days, 7–17 days), *D. biarmipes* (both 3–6), *D. melanogaster* (both 2–9), *D. simulans* (both 2–4), and *D. suzukii* (2–8, 2–6).

Five replicates of each of the following four combinations (i.e., 20 vials) were simultaneously observed for 30 min: a *D. suzuki* male with *D. suzukii* females (control), a *D. suzukii* male with females of one other species, a male of the other species with *D. suzukii* females, and a male and females of the other species (control).

When interspecific copulations were observed, females were subsequently transferred to a vial containing cornmeal diet to check for potential offspring production.

The entire setup was repeated nine times (*D. suzukii* × *D. ananassae*) or eight times (all other combinations), resulting in a sample size of 40 (for *D. melanogaster*, *D. simulans*, and *D. biarmipes*) and 45 (for *D. ananassae*). Because intraspecific *D. suzukii* pairings were present in every setup, the total sample size was 165.

Drosophila males often show species-specific mating behavior (Spieth, 1974). For the species in the present study, this behavior included following the females, different forms of wing display of one or both wings (extending one or both wings, vibrating, scissoring, or flicking), and circling 45°−90° in front of the female (e.g., Mazzoni et al., 2013; Revadi et al., 2015).

Any incidence of interest of a male toward a female (i.e., following or circling the female, any kind of wing display) was noted, as well as attempted or actual copulations. The attempted copulations are of very short duration and can therefore be easily missed (i.e., only a very few were observed). These incidences were added to the "interest" category (see below). When copulation took place, the duration was noted (±20 s). For data representation, the following categories were used: zero incidences of interest → "no interest", one to three incidences of interest → "occasional", four or more incidences of interest → "interest", and for males that copulated → "copulation."

2.4 | Molecular genetic analyses

The identities of *Drosophila* species were confirmed by amplifying and sequencing an internal region of the nuclear glycerol-3-phosphate dehydrogenase (*Gpdh*) marker gene and by subsequent sequence alignment with reference

TABLE 2 Primer combinations used to analyze potential hybrid offspring from the hybridization experiments

Species	Primer (forward, reverse)	Primer sequence	Reference	Fragment length (bp)
D. ananassae	GNL-mel	GTG GTG CCC CAC CAG	Goto et al. (2000)	525
D. melanogaster		TTC AT		526
D. simulans	bia-Gpdh-R	GGG TAG AAG ACG TCC	Kopp and True	526
D. suzukii		ACG AAG CGA ATC AT	(2002)	527
D. biarmipes	bia-Intron-F	ACG GAA AAT TAA AGC CTT TTG CCC CA	This study	335
	bia-Gpdh-R	GGG TAG AAG ACG TCC ACG AAG CGA ATC AT	Kopp and True (2002)	
D. suzukii	suz-Intron-F	ATC AAT CCT TTT GAA ATT TAT TCA CCG CA	This study	338
	suz-Gpdh-R	GGG TAa AAG ACG TCt ACG AAG CGA ATC AT	modified from Kopp and True (2002)[a]	

[a]modifications are indicated in small letters

sequences obtained from the GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA). DNA of individual insects was extracted using the Nucleospin® DNA Insect kit (Macherey & Nagel, Düren, Germany). Primers GNL-mel and bia-Gpdh-R were used to amplify the target region from *D. melanogaster*, *D. simulans*, and *D. ananassae* (Goto et al., 2000; Kopp and True, 2002). *Drosophila biarmipes* and *D. suzukii* were analyzed using GNL-bia: GTt GTG CCC CAC CAa TTt AT (modified from GNL-mel) combined with bia-Gpdh-R. PCRs included Phusion HF Buffer, 7.5 mM $MgCl_2$, 3% DMSO, 0.02 mM dNTP, 0.02 mM of each primer, 0.4 U Phusion Polymerase Hot Start II, ddH_2O, and 2 μl of DNA, resulting in a reaction volume of 20 μl. PCR cycling conditions consisted of 30 s at 98°C, followed by 35 cycles of 5 s at 98°C, 20 s at 62°C, and 1 min at 72°C and a final elongation of 10 min at 72°C.

PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit and sequenced with the primers used for amplification. Sequencing was performed with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and reactions were analyzed with an ABI 3500xL Genetic Analyser (Applied Biosystems, Waltham, MA, USA) equipped with 50-cm capillaries and the POP-7 matrix. Sequences were assembled, manually edited using DNA baser® 4.7.0 software (Heracle BioSoft, Mioveni, Romania), and aligned with eight *Drosophila* spp. reference sequences using BioEdit® 7.0.9 software (Ibis Biosciences, Carlsbad, CA, USA) (for alignment, see Figure S16).

The sequences were submitted to GenBank (accession numbers: OM287431-OM287435).

An assay targeting the *Gpdh* gene region was established to identify potential hybrids between *D. suzukii* and the other four *Drosophila* species as well as between *D. melanogaster* and *D. simulans*. Based on the *Gpdh* sequence alignment, two species-specific primers, bia-Intron-F and suz-Intron-F, were designed for *D. suzukii* and *D. biarmipes*, respectively. Primer pairs shown in Table 2 were used to specifically amplify the target region from different *Drosophil*a spp.

PCRs were performed in volumes of 20 μl and included Promega Flexi Buffer, 1.5 mM MgCl, 0.3% BSA, 0.2 mM dNTP, 0.2 μM of each primer, 1.25 U of Promega GoTaq polymerase, and 2 μl of DNA extract. PCR conditions were the same for all primer pairs and consisted of 2 min at 95°C; 35 cycles of 95°C for 30 s, 62°C for 1 min, and 72°C for 1 min, followed by 5 min at 72°C.

Analyses including the primer pairs (Table 2) matching the two respective parental species were performed in separate reactions for potential hybrids between *D. suzukii* and the other four *Drosophila* species. Subsequent species determination relied on amplification success and fragment-size determined with agarose gel electrophoresis (Figures S2–S5).

Sequence comparison of the assessed *Gpdh* region revealed only six single-nucleotide polymorphisms (SNPs) between *D. melanogaster* and *D. simulans*, confirming the close genetic relationship between the two species. To determine the origin of *D. melanogaster* × *D. simulans* offspring, the *Gpdh* target region was amplified with the primer pairs GNL-mel/bia-Gpdh-R and was sequenced as described above. Hybrid offspring were identified based on the simultaneous detection of both species-specific bases at each of the six SNP positions (Table S1).

3 | RESULTS

3.1 | Hybridization experiments

In the hybridization experiments, *D. suzukii* females and males (five individuals each) were crossed with the opposite sex of the selected four *Drosophila* species (i.e., there were five females or males of *D. suzukii* and five females or males of another species in each replicate vial). Intraspecific crosses of all five species and reciprocal crosses between *D. simulans* and *D. melanogaster* were tested in parallel as positive controls.

In all vials from the intraspecific crosses, offspring emerged, which confirmed that the flies involved were

TABLE 3 Hybrid offspring in the reciprocal crosses between *D. melanogaster* × *D. simulans*

Combination	*n*	Sample No.	Period 1	Period 2	Total females	Total males	Total offspring
D. melanogaster (f) × *D. simulans* (m)	48	3	0	38	38	0	38
		4	0	1	1	0	1
		26	0	37	37	0	37
		39	6	29	35	0	35
D. simulans (f) × *D. melanogaster* (m)	48	17	43	4	11	36	47
		21	0	1	0	1	1
		36	0	16	5	11	16
		39	49	56	21	81	105[a]

Note: *Drosophila* pairs (m-males, f-females) were provided fresh diet to oviposit for two periods of 4 days each.
[a]Three of the offspring could not be sexed.

capable of reproduction (Table 1; Figure S1). Within an 8-day period, the mean (±SE) number of offspring produced by the five pairs ranged from 223 ± 13.3 (*D. biarmipes*) to 742 ± 35.2 (*D. melanogaster*).

In the *D. melanogaster* × *D. simulans* crosses, hybrids emerged from four of 48 replicates in both male × female combinations (Tables 1 and 3). We assume that in each of the total of eight replicates, only one of the five females reproduced because the maximum offspring number of 105 in one of the replicates (Table 3) is within the range produced by a single *D. simulans* female during 8 days (Figure S1). It follows that only 1.7% of the females produced hybrid offspring. The hybrid origin for 13 randomly selected offspring was confirmed with a molecular genetic approach established in this study (see below) (Table S1). The viability and fertility of the hybrids were not assessed.

In 334 of the total 336 interspecific combinations involving *D. suzukii* with one of the four other *Drosophila* species, no offspring emerged (Table 1). In the two replicates in which offspring emerged, molecular analyses confirmed that they were not hybrids. In the first replicate (*D. ananassae* males × *D. suzukii* females), one offspring (female) was found and was subsequently identified as *D. ananassae* based on morphological as well as molecular analyses (Figure S2). This can only be explained by the scenario that this individual having been placed into the test container erroneously. In the second replicate (*D. biarmipes* males × *D. suzukii* females), a total of 31 offspring emerged. Molecular genetic analyses confirmed that all of the offspring were *D. suzukii* (Figures S3 and S4). We inferred that, in this sample, one *D. suzukii* female must have been mated before the experiment.

3.2 | Male mating behavior

In this experiment, the mating behavior of one male fly was observed when placed together with three females, so that there were four flies in the mating arena. *Drosophila suzukii* females and males were combined with the opposite sex of the other four *Drosophila* species. Intraspecific combinations served as a control. The age of the individuals differed among species and was chosen to ensure sexual maturation.

In all intraspecific combinations, males were interested in (indicated by following or circling the female or wing display) and copulated with females, though to different degrees (Figure 2a). *Drosophila suzukii* males were interested in *D. ananassae* females in only three of 45 cases, but they showed stronger interest in the females of the other three species (Figure 2b). In combination with *D. melanogaster* and *D. simulans* females, *D. suzukii* even achieved copulations, ranging in duration from 10 s (with a *D. melanogaster* female) to 11 min (with a *D. simulans* female). *Drosophila ananassae* and *D. biarmipes* males showed no interest in *D. suzukii* females (Figure 2c). Males of *D. melanogaster* and *D. simulans*, in contrast, frequently showed interest in *D. suzukii* females but did not achieve copulation.

When interspecific copulations were observed, females were checked for offspring production. In one of the samples in which a *D. suzukii* male copulated with a *D. simulans* female, four offspring emerged, and their genotype was screened. All four specimens were identified as *D. simulans* (Figure S5). It follows that the female must have mated in the short period before it was used in the experiment.

3.3 | Molecular genetic analyses

An assay was developed to confirm the identity of potential hybrid offspring by using specific combinations of primer pairs targeting an internal region of the nuclear glycerol-3-phosphate dehydrogenase (*Gpdh*) marker gene (Table 2). For the positive hybridization control (*D. melanogaster* × *D. simulans*), one primer pair that amplifies the target region in both species was used. PCR products were sequenced, and the simultaneous detection of species-specific bases at six single nucleotide polymorphic (SNP) sites was used to identify hybrid offspring (Table S1). For all interspecific crosses involving *D. suzukii*, two primer pairs with different species-specificity (Table 2) were used, which allowed determination of offspring origin based on positive/negative amplification and fragment length (Figures S2–S5).

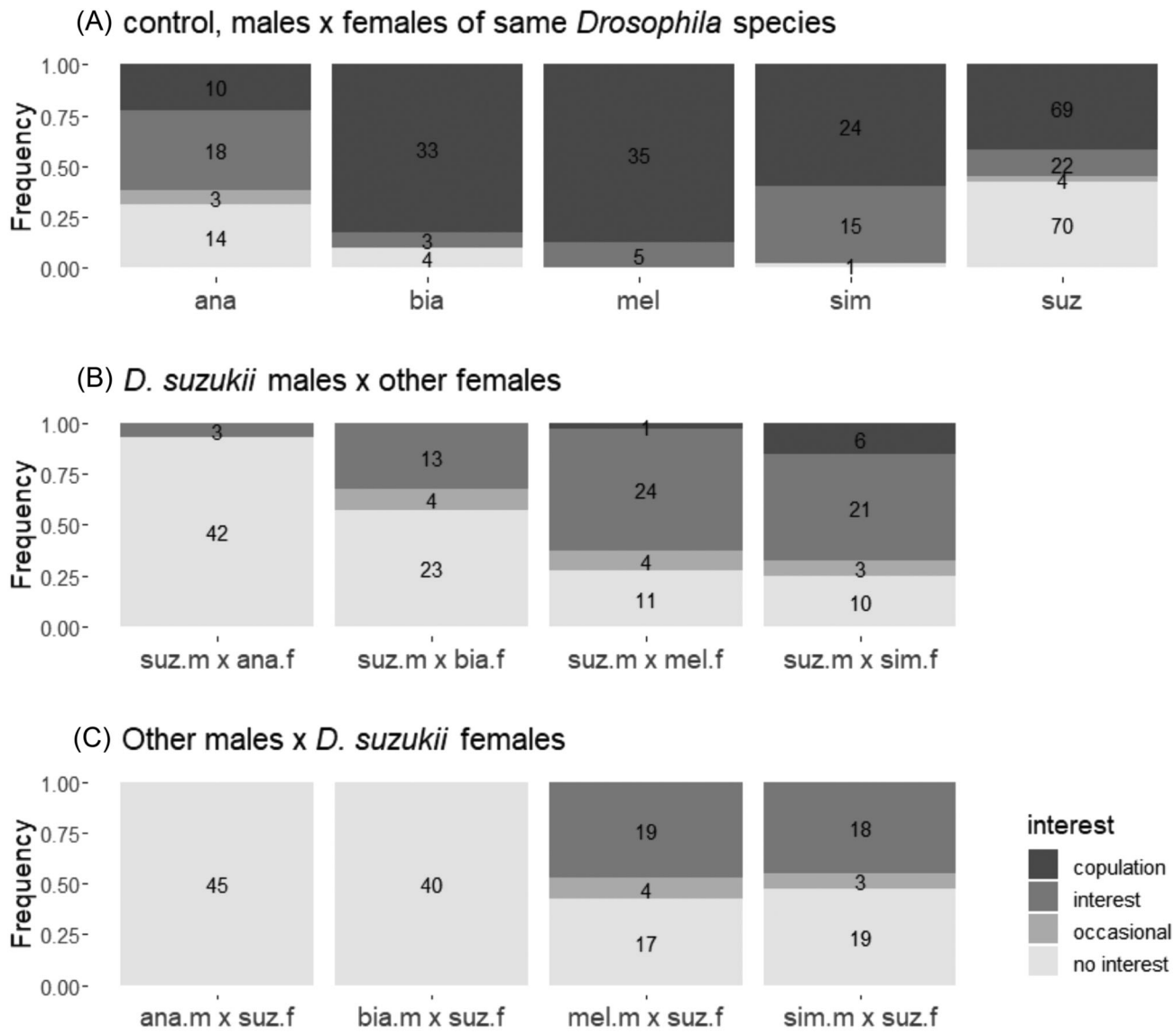

FIGURE 2 Male mating behavior of 13 combinations of intra- and interspecific crosses: (a) intraspecific behavior (controls), (b) *D. suzukii* (suz) males (m) × females (f) of *D. ananassae* (ana)/*D. biarmipes* (bia)/*D. melanogaster* (mel)/*D. similans* (sim), and (c) males of *D. ananassae*/*D. biarmipes*/*D. melanogaster*/*D. simulans* × *D. suzukii* females. One male was combined with three females and observed for 30 min for incidences of interest and copulations. If only one to three incidences of interest were observed, this was considered "occasional." Four or more incidences of interest were considered as mating "interest." The numbers in the bars indicate the number of males recorded for each category.

4 | DISCUSSION

Using *D. suzukii* as a case study, we demonstrate that the potential for hybridization can be assessed by (i) identifying the taxonomically most closely related nontarget species, (ii) conducting interspecific hybridization experiments under controlled laboratory conditions, (iii) unambiguously identifying hybrid offspring through molecular genetic analyses, and (iv) observing mating behavior in interspecific pairings. Overall, our study revealed no indication that *D. suzukii* will hybridize with *Drosophila* species in regions in Central Europe invaded by the pest.

In our hybridization experiments, none of the crosses between *D. suzukii* and the three taxonomically most closely related *Drosophila* species occurring in Europe (*D. ananassae*, *D. melanogaster*, and *D. simulans*) resulted in hybrid offspring. Neither did the crosses with *D. biarmipes*, a species of the *D. suzukii* subgroup occurring in Southeast Asia but exotic to Europe, resulted in hybrid offspring. Although a few putative offspring were found in some crossings, molecular genetic analyses confirmed that the offspring were not hybrids but were artifacts apparently caused by accidental handling errors of the morphologically similar *Drosophila* species. This underlines the relevance of using molecular genetic identification methods to provide unambiguous results when working in large-scale assays with closely related species that cannot be easily distinguished otherwise. Here, the hybridization experiments were suitable to

produce hybrid offspring as demonstrated by the positive control (i.e., interspecific crosses of *D. melanogaster* × *D. simulans*). When female *D. melanogaster* were crossed with male *D. simulans*, only female hybrid offspring emerged. However, when *D. simulans* served as females, hybrid offspring of both sexes emerged, albeit with a male bias. These results are in line with previously reported ones and confirm that the degree of mating success between the two species can vary among strains (Watanabe et al., 1977; Sawamura, 2000).

A limitation of our experiments is that the occurrence of rare hybridization events might not be detected. It happens despite the fact that we have increased the probability of interspecific matings by selecting male and female flies at the optimum age and by combining them in small vials in the absence of their natural mating partners. We therefore observed the behavior of interspecific *Drosophila* spp. pairs, including either male or female *D. suzukii*, to assess whether they actually recognize each other as potential mating partners. *Drosophila* spp. show a highly complex courtship behavior and rely on visual, chemosensory, and auditory signals for sexual interactions; such signals are species-specific and differ between males and females (Spieth, 1974). The mating behavior of *D. suzukii* differs in some aspects from that of other species in the *D. melanogaster* species group (Revadi et al., 2015). For example, in contrast to most other species, *D. suzukii* does not use the male-produced cis-11-octadecenyl acetate (cVA) as a volatile sex pheromone (Dekker et al., 2015) and relies on substrate-born vibrations (Mazzoni et al., 2013). According to our observations, male *D. suzukii* showed a considerable interest in females of *D. melanogaster* and *D. simulans*. Moreover, *D. melanogaster* and *D. simulans* males were also interested in *D. suzukii* females. In the case of *D. suzukii* males, interspecific copulation attempts were observed in a few cases, but they did not result in hybrid offspring. In the case of *D. ananassae* and *D. biarmipes*, behavioral observations confirm that the potential of hybridization with *D. suzukii* is highly unlikely.

Even though we combined hybridization experiments with behavioral observations, we cannot exclude the possibility of rare hybridization events for a number of reasons. First, the number of tests that can be conducted is limited such that a rare event might not be observed. According to the "rule of three" (Hanley and Lippman-Hand, 1983), we could only have detected hybridizations events that occur at a rate of 1.2%–1.4% (considering 42 to 52 vials with five females and five males each) with 95% confidence. To detect a rarer event that occurs, for example, at a frequency of 0.1%, one would already have to include 600 vials containing five mating pairs each. Second, the hybridization success might differ among strains (Sawamura, 2000), and third, testing under confined conditions might not sufficiently mimic the natural conditions (micro-habitat, age of the flies, environmental conditions, etc.). Additional uncertainty comes from the fact that climate change might affect species distribution, interactions and behavior that could lead to successful hybridization events in the future (Larson et al., 2019).

As described in detail for GE plants (Raybould and Cooper, 2005; Devos et al., 2018), it is important to recognize that, in a risk assessment context, rare hybridization events with geGD organisms do not necessarily lead to harm. For such harm to occur, the hybrid offspring must be viable and fertile. In addition, the GD must lead to population suppression or modification in the recipient organism, and a protection goal must be affected. Although we can increase certainty in a "no-hybridization" conclusion by adding more experiments and observations, rare events cannot be excluded. Thus, some uncertainty will always remain. It is therefore necessary that risk managers define thresholds above which harm is indicated or additional studies are required. This threshold of course depends on the category of GD, that is, whether or not it is self-sustaining and whether it is a high- or low-threshold GD. Modeling approaches used to describe the spread of GD elements in a population (e.g. Marshall et al., 2017; Sánchez et al., 2020) can help to assess the impact of rare hybridization events on nontarget *Drosophila* populations. Brown et al., 2022, for example, have demonstrated how probabilistic estimates for potential harm caused by the environmental release of geGD organisms can be derived using the Bayesian network-relative risk model.

Remaining uncertainties might also be addressed by post-release monitoring to confirm the ERA assumptions and to gather additional data that might trigger an action and/or feed back into ERA for future products. Such post-release monitoring is a regulatory requirement for GE organisms, including GE insects, in some jurisdictions and is also required or recommended for the release of exotic biological control agents (Romeis et al., 2020; Devos et al., 2022).

Although our case study concerned Central Europe, the results are also relevant for other regions of the world in which *D. suzukii* is invasive and where the release of a geGD *D. suzukii* strain might be considered to control this invasive pest. The *D. suzukii* species subgroup of the *D. melanogaster* species group contains 17 species, most of which are present in the Oriental and/or Palearctic regions with only two species in the Afrotropical and two in the Australasian region (Brake and Bächli, 2008). *Drosophila suzukii* is the only species of this subgroup currently present in Europe and the Americas (Brake and Bächli, 2008; Bächli, 2022). Thus, in the invaded regions, the taxonomically closest relatives of *D. suzukii* are in the *D. melanogaster* species group. As is the case in Europe, only the three cosmopolitan species, *D. ananassae*, *D. melanogaster*, and *D. simulans*, are present in North America (Brake and Bächli, 2008). In South America, a fourth species, *D. malerkotliana*, has been reported (Brake and Bächli, 2008). The situation, however, is different in regions such as Asia, where other species in the *D. suzukii* species subgroup are present and where additional hybridization studies with those species may be warranted before considering the release of a geGD *D. suzukii* strain.

Evidence for hybridization between *D. suzukii* and another species from the *D. suzukii* subgroup (*Drosophila subpulchrella*) has been reported from the species' area of origin. In Asia, the two species have a highly overlapping geographical

distribution (Bock and Wheeler, 1972) where they can coexist in sympatric locations and use similar resources, that is, ripening fruits (Mitsui et al., 2010). In the laboratory, crosses between *D. suzukii* and *D. subpulchrella* led to some fertile offspring (Fuyama, 1983) (note that *D. subpulchrella* was erroneously named *Drosophila pulchrella* in that paper; Muto et al., 2018) even though the two species are not only morphologically distinct but also qualitatively differ in their courtship behaviors (Fuyama, 1983). Studies on *Wolbachia* infections indicate that hybridization of *D. suzukii* with *D. subpulchrella* has also occurred in the field (Conner et al., 2017).

To minimize the risk that an engineered GD transfers to nontarget populations of *D. suzukii* or to nontarget species in general, more localized geGDs should be deployed that are self-limited or that only spread if present above a certain threshold frequency (L. S. Alphey et al., 2020; Devos et al., 2021). In laboratory studies, promising results have already been obtained with such geGDs for *D. melanogaster* (e.g., Buchman et al., 2021; Terradas et al., 2021).

5 | CONCLUSIONS

We have demonstrated that the potential for hybridization of a hypothetical geGD *D. suzukii* strain and nontarget *Drosophila* species in invaded regions will be limited. We also offered recommendations on how to assess the potential of such hybridizations in support of case-specific ERA by a combination of interspecific hybridization experiments, behavioral observations, and molecular genetic analyses.

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DATA AVAILABILITY STATEMENT

The data used in this study are available at https://doi.org/10.5061/dryad.z34tmpghs.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Box 1. Target/non-target terminology

Target species	– Insect species targeted by the engineered GD (e.g., *Drosophila suzukii*)
Target population	– Population of the target species to be suppressed or modified (*D. suzukii* in Europe)
Non-target population	– Population of the target species that should not be affected by the GD (*D. suzukii* in the area of origin, i.e., East Asia)
Non-target species	– Species (and all of its populations) that should not be affected by the GD (e.g., other *Drosophila* species, antagonists of *D. suzukii*)