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Managing and monitoring genetic isolation and local adaptation of endemic and introduced *Cotesia sesamiae* for the biological control of the cereal stemborer *Busseola fusca* in Cameroon

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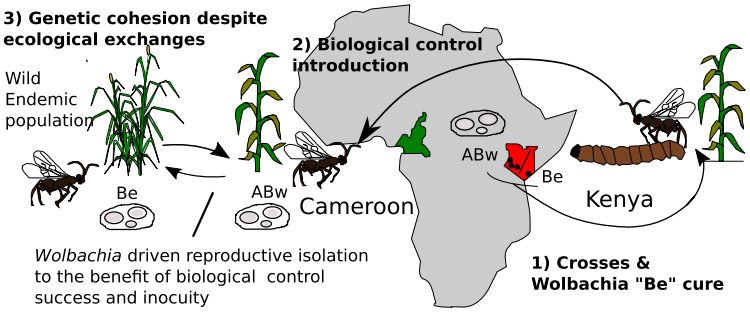
HIGHLIGHTS

- *Cotesia sesamiae* was redistributed from Cameroon to Kenya to control maize pests.
- Success needed coexistence with wild host plants endemic conspecifics in dry season.
- *Wolbachia* bacteria limited gene flow to protect endemic genetic adaptations.

GRAPHICAL ABSTRACT



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ABSTRACT

The success of biological bontrol (BC) introductions can be enhanced by considering theory and knowledge of biological systems. The gregarious braconid parasitoid *Cotesia sesamiae* (Cameroon) is one of the best studied biological control agent from the perspective of molecular ecology. Its evolutionary adaptation to the target host involves symbiotic partners. Polydnaviruses are responsible for immune and developmental adaptations whereas *Wolbachia* bacteria may reinforce this local adaptation though genetic isolation mechanisms. The noctuid *Busseola fusca* is a major stemborer pest of maize in sub-Saharan Africa. In contrast to eastern Africa, *C. sesamiae* is rarely found on *B. fusca* in western Africa. It is however often obtained from other stemborer species feeding on wild grasses. A biological control project was launched in 2006–2007 by introducing to Cameroon seven crosses of Kenyan populations of *C. sesamiae* collected in different ecozones. They included populations adapted to *B. fusca* that develop on maize as well as populations adapted to other hosts feeding on wild plants to allow

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carryover between cropping seasons. *Wolbachia* strains responsible for cytoplasmic reproductive incompatibilities with endemic strains were included in the crosses to limit genetic exchanges between introduced and endemic *C. sesamiae* and preserve genetic adaptation to *B. fusca* of the introduced populations, while at the same time preserving their ability to survive on wild plants. Six post release surveys were carried out on maize from 2007 to 2013, and on wild grasses in 2013. A total of 393 *C. sesamiae* individuals, each from one cocoon mass, were genotyped for 11 microsatellite loci. Multidimensional scaling analysis, STRUCTURE and GENECLASS analyses assigned almost all the parasitoids recovered from maize to those introduced from Kenya. The introduced strains were also recovered from wild host plants with little genetic exchanges with endemics. Each population remained strongly associated with its original *Wolbachia* component, suggesting that *Wolbachia* may contribute to genetic isolation between endemics and introduced populations in wild host plants when maize is absent, thereby conciliating biological control success and safety.

1. Introduction

Classical Biological control (BC) operations can be optimized using evolutionary ecology knowledge, which should also minimize the risks toward non target organisms (Szűcs et al., 2019). In contrast to chemicals or genetically modified organisms, biological control agents can theoretically evolve to overcome the evolution of resistance in the host (Holt and Hochberg, 1997). There is empirical evidence of evolution in BC which can affect its control success (Szűcs et al., 2019). In addition to adaptation to target species, BC agents can evolve adaptation to non targets which can potentially endanger endemic biodiversity (Simberloff, 2012; Le Hesran et al., 2019). Research aiming at understanding evolutionary ecology of host specialization has been considered as a particularly essential aspect of the efficiency and safety of BC (Brodeur, 2012).

In Cameroon, indigenous parasitoids do not exert sufficient control over the predominant stemboring maize pest *Busseola fusca* (Fuller) (Lepidoptera, Noctuidae) (Ndemah et al., 2001, 2003, 2007). A comparison between regions of Africa showed that certain species and populations of parasitoids are lacking in this country and in western Africa in general (Schulthess, 1997). The gregarious braconid *Cotesia sesamiae* (Cameron) is one of the most studied biological control agents in terms of its ecology and genetics (Kaiser et al., 2015, 2017). It is commonly found on *B. fusca* in East and Southern Africa. By contrast, in Cameroon it was found only on noctuid stemborer species feeding in wild grasses but never on *B. fusca* on maize (Ndemah et al., 2001, 2007).

Adaptation to *B. fusca* in Kenya involves immune suppression mediated by symbiotic polydnaviruses (PDV) found in the calyx fluid of the parasitoid ovary, which is injected into the insect host together with the eggs during oviposition (Ngi-Song, 1998; Gitau et al., 2010). PDVs are directly related to parasitism success, enabling the parasitoid to overcome the immune system of its host (Gitau et al., 2010; Herniou et al., 2013). They disable and prevent host haemocytes from encapsulating parasitoid eggs (Gitau et al., 2010). The difference in virulence of *C. sesamiae* populations on *B. fusca* is correlated with differences in expression of the nucleotide sequence CrV1 of the parasitoid PDV gene (Gitau et al., 2007). Branca et al. (2011) reported polymorphism in PDV haplotypes across Africa. In western Kenyan *C. sesamiae*, the PDVs are virulent against *B. fusca*, while in Cameroon the PDVs are not virulent against *B. fusca*. Further DNA analysis on a larger numbers of host species and on a wider geographic range confirmed the association between the hosts in which the parasitoid had developed, and the genotype of the PDV CrV1 gene (Branca et al., 2011).

Cotesia. sesamiae populations are also infected by different strains of *Wolbachia* spp. responsible for cytoplasmic incompatibilities when intercrossed, and potentially reinforcing specialization and local adaptation to different hosts (Branca et al., 2009). Incompatibilities can be unidirectional (when infected males mate with a non-infected females) or bidirectional (through matings between insects harboring different strains of the bacterium). Four *Wolbachia* variants have been identified from *C. sesamiae* collected in Kenya on maize (Mochiah et al., 2002; Gounou et al., 2008; Branca et al., 2019). In western Kenya, the parasitoid developing on *B. fusca* is infected by the *Bwest* variant, while in the eastern coastal region the parasitoid developing on the noctuid cereal stemborer *Sesamia calamistis* Hampson, it is infected by the *A*, or *Beast* strain or co-infected by *A* + *Beast strains*. In hybridization experiments, the number of females in the offspring is reduced in both directions of mating. Incompatibility is total for ♀*east* × ♂*west* and weak for ♀*west* × ♂*east* (Branca et al., 2019). Branca et al. (2019 supplementary data) estimated an infection rate of 92% across Africa, which somewhat reduces incompatibility.

Because of the differences in the ecology and economic importance of *C. sesamiae* on *B. fusca* in Cameroon and eastern Africa, a BC project to redistribute Kenyan *C. sesamiae* populations on *B. fusca* in Cameroon was proposed by Schulthess et al. (1997). Non-target effects were assessed through detailed studies on other borer species occurring in wild habitats as well as on associated *Wolbachia* and PDVs (Ndemah et al., 2007; Gitau et al., 2007, 2010; Dupas et al., 2008; Branca et al., 2009, 2011). *Busseola fusca* is the target species but wild stemborers enable the parasitoid to over-season when green maize stalks are not available and *B. fusca* is diapausing in dry maize stalks. There was a trade-off between the necessity to maintain introduced parasitoid populations on neighboring wild habitats and the need to prevent non target impact on endemic parasitoid populations. In Cameroon, endemic *C. sesamiae* in wild habitats are infected by the *A* or *A* + *Beast Wolbachia* strains (Branca et al., 2009). Interestingly the incompatibility of the Cameroonian strains is stronger than that of the Kenyan strains (Branca et al., 2019); therefore, differences in *Wolbachia* strains could be used to protect endemic Cameroonian populations from hybridization with introduced Kenyan strains.

Using knowledge on polydnavirus and *Wolbachia* molecular ecology, emphasis was given in this project to introducing strains that (1) maximize impact on crops, by harboring genes adapted to *B. fusca* and maize, (2) can perennate the off-season on other stemborers attacking wild plants, by harboring some genes adapted to wild host, but (3) do not interbreed with endemic *C. sesamiae* present on these plants, by harboring *Wolbachia* strains different from the endemics to avoid non-target impact.

The specific objectives of the project were: (1) to select and cross strains expected to maximize BC success while minimizing ecological side effects to Cameroonian *C. sesamiae* and wild borers; and (2) to survey the genetic material of endemic and introduced strains in wild and cultivated habitats and their relationship with *Wolbachia* strain status to test the predictions of establishment of Kenyan populations on *B. fusca* on maize and survival on wild host plants without gene flow from introduced to endemic strains of *C. sesamiae*.

2. Materials and methods

Constitution of Kenyan *Cotesia sesamiae* populations for introduction in Cameroon: Role of *Wolbachia*

The parental populations of *C. sesamiae* were collected in Kenyan maize fields in Kitale (Ki), Meru (Me), and Kakamega (Ka) from *B. fusca*, in Mount Kenya 1 (MK1) and Mombasa (Mb) from *S. calamistis*, and in Tana River (Ta) from *Sesamia firmata* Moyal infesting the Napier grass *Pennisetum purpureum* Schumach (Fig. 1, Suppl. Table 1). Maize or

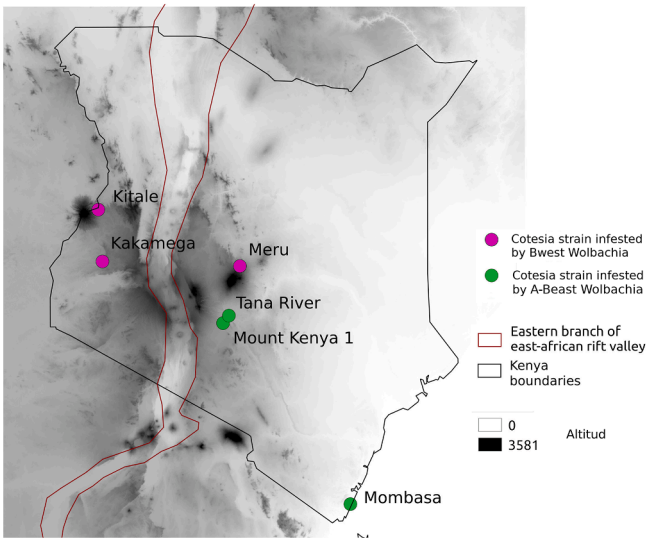

Fig. 1. Populations collected in Kenya and their *Wolbachia* strain infection status.

P. purpureum plants that exhibited signs of stem borer feeding were collected and dissected. Borer larvae and cocoons of *C. sesamiae* were placed individually in glass vials (7.5 cm × 2.5 cm). The larvae were provided with a piece of maize stem or artificial diet (Onyango and Ochieng'-Odero, 1994). A total of 395 cocoon masses were collected. The number of cocoon masses collected per locality is given in suppl. Table 1. The larvae and cocoons were kept in the laboratory at *icipe* (African Insect Science for Food and Health, Nairobi, Kenya) and observed for cocoon formation and parasitoids emergence. Adult *Cotesia* spp. that emerged from cocoons were identified using the shape of the male genitalia or female propodia (Kimani and Overholt, 1995) at *icipe*'s biosystematics unit. *Cotesia sesamiae* progeny were allowed to mate in a vial for at least 2 h. under a strong source of light Because of lack of a rearing protocol, *C. sesamiae* from Tana River were reared on *S. calamistis* instead of their original host, *S. firmata*.

2.1. Parasitoid rearing

Adults were fed a 20% honey solution. After exposing the larvae to the parasitoids using the hand stinging method by Overholt et al. (1994), the parasitized larvae were placed on artificial diet at 26 ± 1 °C until cocoon formation and parasitoids emergence (Onyango and Ochieng'-Odero, 1994). Time to cocoon formation took 12 to 14 days. The progeny that emerged were used to start the F1 colony for mass production in the laboratory. Fifteen, 14, 2, 2, 10 and 13 cocoon masses were used to initiate the Cs-Ki, Cs-Me, Cs-Ka, Cs-MK1, Cs-Mb and Cs-Ta colonies, respectively.

2.2. Wolbachia curing

The aim was to produce Kenyan strains for release that were either not infected or infected by a *Bwest Wolbachia* strain, not present in Cameroon (Branca et al., 2019). The variant of *Wolbachia* present in each strain was identified with the *wsp* gene AFLP test (Branca et al., 2011) using two individuals per population, before and after mass production in the laboratory. The Ki, Me and Ka populations that were infected with the *Bwest* variant of the bacterium were not cured of *Wolbachia*. The populations infected by both the *A* and *Beast* were cured from *Wolbachia* using a rifampicin (Sanofi-Aventis, France) treatment for one generation (Mochiah et al., 2002). The absence of *Wolbachia* after treatment was confirmed by PCR test on DNA extracts.

2.3. Crosses for constructing hybrid release strains

Hybrid populations were produced by crossing populations infected by the same *Wolbachia Bwest* strain (i.e., Me, Ki and Ka) or cured from the *A*, *Beast* and *A/Beast* strains present in Cameroon (i.e., MK1, Ta and Mb) (Table 1). Each individual cocoon was placed in a vial. Freshly emerged males and females from the different populations were selected and observed to cross-mate. Mated females were offered *B. fusca* larvae for parasitism using the hand stinging method.

The first hybrid population (named Hy) was initiated from reciprocal crosses between Cs-Ki and Cs-Me, after 11 and 9 generations of rearing in the laboratory, respectively (Table 1, 20 males and 20 females for each cross). The progenies of the reciprocal hybrids resulting from each cross were then mixed to obtain the Hy population, and then reared for 1 generation before being sent to Cameroon.

The second hybrid population named Ke1 resulted from crosses between Ki, Me and Ka (Table 1). The progenies of the hybrids resulting from each cross were then mixed to obtain the Ke1 population and then reared for 1 generation before being separated for cured and uncured procedures (see below).

The third hybrid named Ke2 resulted from the cross between all six populations (MK1, Ta, Mb, Ka, Me and Ki) (Table 1). All reproductively compatible crosses (males not infected by *Wolbachia*) were realized. The progenies were then mixed to obtain the Ke2 population and reared for 1 generation before being separated for cured and uncured procedures (see below).

Ke1 and Ke2 hybrid population were cured of *Wolbachia* using the rifampicin protocol described above (Mochiah et al., 2002) to obtain the $Ke1^{W-}$ and $Ke2^{W-}$ populations.

Considering the cross proportions described above and in Table 1, the genetic material introduced in the Hy, Ke1 and Ke2 populations

Table 1
Constitution of the Hy | Cs-Kenya 1 | Cs-Kenya 2 release strains. The column name is the strain of the father (F.) and the line name, the strain of the mother (M.) . The number of parental pairs for each introduced populations are given in the following order: Hy | Cs-Kenya 1 | Cs-Kenya 2 (Hy|K1|K2). Parental strains are: Ki: Kitale, Me: Meru, Ka: Kakamega, MK1: Mount Kenya 1, Ta: Tana River, Mb: Mombasa. Total G: total genetic contributions of each parent in the cross. Wb: *Wolbachia* variants: WE: *Wolbachia* variants found endemically in cameroon (A and Beast strains, Branca et al., 2019). WI: *Wolbachia* variants not found endemically in cameroon (Bwest strain, Branca et al., 2019). *: the strains harboring WE were cured from *Wolbachia* before the crosses so that the resulting Cs-Kenya 1 and Cs-Kenya 2 strains were WI + WE (infected by WI and cured from WE). Insect: species of insect host from which the strain was collected in Kenya: *B. fusca: Busseola fusca, S. calam.: Sesamia calamistis, S. firm.: Sesamia firmata.* Plant: plant species from which the insect was collected in Kenya: *P. purp.: Pennisetum purpureum. Z. mays: Zea mays.* Superscripts ^{Bf}: ratio of genetic contribution of genes sampled on *B. fusca*. ^{Zm}: ratio of genetic contribution of genes sampled on *Z. mays* versus on wild host plant *P. purpureum*.

M.\F.	Ki	Me	Ka	MK1	Mb	Ta	Total G Hy|K1|K2	Wb	Insect	Plant
Ki	0|7|5	30|7|5	0|7|5	0|0|0	0|0|0	0|0|0	**20|42|30**	WI	*B. fusca*	*Z. mays*
Me	30|7|5	0|7|5	0|7|5	0|0|0	0|0|0	0|0|0	**20|42|30**	WI	*B. fusca*	*Z. mays*
Ka	0|7|5	0|7|5	0|7|5	0|0|0	0|0|0	0|0|0	**0|42|30**	WI	*B. fusca*	*Z. mays*
MK1	0|0|1	0|0|1	0|0|1	0|0|0	0|0|0	0|0|0	**0|0|6**	WE*	*B. fusca*	*Z. mays*
Mb	0|0|3	0|0|3	0|0|3	0|0|0	0|0|0	0|0|0	**0|0|18**	WE*	*S. calam.*	*Z. mays*
Ta	0|0|3	0|0|3	0|0|3	0|0|0	0|0|0	0|0|0	**0|0|18**	WE*	*S. firm.*	*P. purp.*
Total G	**20|21|22**	**20|21|22**	**0|21|22**	**0|0|0**	**0|0|0**	**0|0|0**	**x|63|66**		**1|1|.86^{Bf}**	**1|1|.93^{Zm}**

originated at 100%, 100% and 89%, respectively, from individuals collected on *B. fusca* larvae the rest being collected on *Sesamia calamistis* and *Sesamia firmata*, and at 100%, 100% and 93% from individuals collected on maize, the rest being collected on *Pennisetum purpureum*. The aim was to introduce populations harboring genes permitting to counteract *the* immune reaction of *B. fusca* (Dupas et al., 2008) and genes permitting to switch host plant and host species.

In total, two parental (Ki, Me) and five hybrid populations (Hy, Ke1 and Ke2 infected by *B west Wolbachia*, and Ke1^{W-} and Ke2^{W-}, cured from *Wolbachia*) were released in Cameroon.

2.4. Releases of parasitoids in Cameroon

The releases were conducted in 193 sites from 44 localities between 2006 and 2008. At each site a specific strain was released (Fig. 2, suppl. material for details). First releases were conducted in inland valleys (wetlands) maize fields of the forest zone, during the off-season. Cocoon masses were packed into boxes with cotton wool and sent under ambient environmental conditions *via* express air mail from Nairobi to the International Institute of Tropical Agriculture (IITA) in Yaoundé, where they were kept at room temperature (26 °C ± 2) in vials with cotton wools.

In 2006–2007, the five (Hy, Ke1U, Ke2U, Ke1C, Ke2C) hybrids as well as the two parental (Ki, Me) populations of *C. sesamiae* were released as mated adult females. Infested larvae, received from Kenya in a 1 L plastic jar covered with a fine insect proof screen mesh, were inspected daily for adult emergence. Adults were fed with a 20% honey solution soaked in cotton wool. They were allowed to mate for three hours under artificial light in the laboratory. Adults emerging from a single cocoon mass were released in each of 147 fields in 11 villages in the low altitude forest zone and in high altitudes zone with the agreement of the farmers (Fig. 2a).

In 2007–2008 the same strains (Ki, Me, Hy, Ke1U, Ke2U Ke1C, and Ke2C crosses) were released as parasitized *B. fusca* larvae in tunnels drilled into maize stem, in 119 maize fields in 42 localities, in the forest zone and high altitudes zone. The longitude, latitude and altitude of the release localities (Fig. 2a) as well as the number of parasitized larvae released were recorded (Suppl. material).

2.5. Surveys in maize fields

Post release surveys were conducted in 2007, 2008, 2009, 2010, 2011 and 2013 in the release villages. In the high altitudes, there is one long maize season from March to October while in the forest zone there are two to three maize seasons, two during the two rainy seasons from March to June and from end of August to early December, respectively, and a last one during the dry season (January to April). The fields in the forest zone were surveyed mostly during the second rainy season in October/November, when pest densities are expected to be high, while in the high altitude most of the fields were surveyed in June/July when *B. fusca* is in the right stage for parasitization by *C. sesamiae*. Surveys were also conducted in Yaoundé where the parasitoid might have escaped from the lab and invaded farmers' fields.

Each field was divided into four quadrants, and six maize plants per quadrant were randomly sampled. In addition, four plants per quadrant that showed borer damage were sampled. During 2013, only three infested plants were sampled per quadrant. In each field, the maize plants sampled were dissected and checked for stem borer larvae and pupae, and *C. sesamiae* cocoon masses. The larvae found were reared on stem and cob pieces of maize in the lab until emergence of adult moths or parasitoid cocoon formation. Each cocoon mass was placed in a separate transparent plastic container. Once the adults had emerged and died, the number of cocoons per mass, the total number of emerged adults as well as the number of females and males were counted. The adult parasitoids were preserved in 95% ethanol and sent to the Institut de Recherche pour le Développement (IRD) lab at Gif Sur Yvette in France for molecular analyses. The post-release sites surveyed are shown in Fig. 2b.

2.6. Surveys on wild host plants

Before releasing the Kenyan populations of *C. sesamiae* in Cameroon, pre-release surveys were conducted on four wild host plants, namely *Pennisetum purpureum* (Schumach), *Setaria megaphylla* (Steud.) Dur. & Schinz, *Setaria* spp. and *Typha domingensis* (Table 2). Surveys for *C. sesamiae* on wild noctuids and *B. fusca* were conducted in roadside patches of wild grasses in the forest zone and highlands of Cameroon

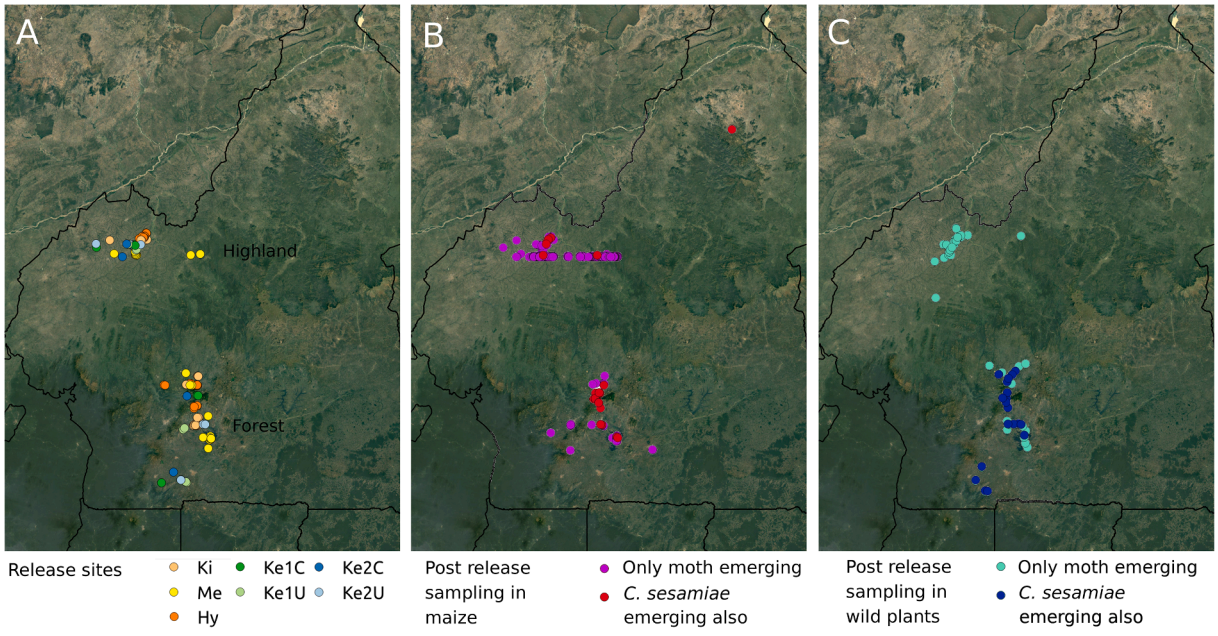

Fig. 2. Release and recoveries of *C. sesamiae* from maize and wild host plants in highlands (northern points) and forest Zone (southern points) of Cameroon. A: Release sites on maize. B: post release sampling on maize. C: Post release sampling on wild host plants. Me: Meru, Ki: Kitale, Hy: Me × Ki, Ke1: Meru × Kitale × Kakamega, Ke2: Meru × Kitale × Kakamega × Muranga × Mombasa × Tana River. C: Cured from *Wolbachia*. U: Uncured from *Wolbachia*. × : crossing.

Table 2
Total number of *Cotesia sesamiae* cocoon masses sampled before and after releases, according to plant and insect hosts.

Host plant		Insect host	No of cocoon masses collected	
			Pre-release	Post-release
Cultivated	*Zea mays* (L)	*Busseola fusca* and *Sesamia calamistis*	0	96
Wild	*Pennisetum purpureum* (Moench)	*Poeonoma serrata*	5	83
	Setaria megaphylla (Steud.) Dur. & Schinz	Undetermined	1	1
	Setaria sp	*Sesamia* sp	2	0
	Typha domingensis Pers.	Undetermined	1	0
	Undetermined	Undetermined	0	12
	Total		**9**	**192**

between 2001 and 2003 (Ndemah et al., 2007; Branca et al., 2011). After release, in 2013, wild noctuids were collected from grasses at the edges of maize fields in the sites as shown in Fig. 2c and prepared for molecular analysis.

Analysis of *Cotesia sesamiae* specimens for microsatellite markers and *Wolbachia* infection

All the insects emerging from larvae collected during the surveys were preserved in alcohol. From each cocoon mass emerging, one individual was analyzed for 11 microsatellite loci (Jensen et al., 2002; Abercrombie et al., 2009; Branca et al., 2019) and *Wolbachia wsp* loci (Branca et al., 2011). DNA of was extracted individually using the NucleoSpin Tissue XS (Macherey-Nagel) following the manufacturer's protocol. Next, approximately 10 ng of DNA was added to 5 μl of 2X Platinum Multiplex PCR Master Mix (Life Technologies), 1 μl of Primer mix (final concentration of 0,1 μM for each primer) and 3 μl of water in a PCR to amplify. The list of primers and amplification protocol are given in supp. Table 2 The PCR was carried out at a denaturation temperature of 95 °C for five minutes, then 25 or 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by a final elongation of 5 min at 72 °C. PCR products were then run on an ABI 3130 XL sequencer at the IRD lab at Gif-sur-Yvette in France, and the profiles analyzed with GeneMapper v3.1 (Life Technologies).

Wolbachia strain infection was detected by amplifying the *wsp* gene using the protocol developed by Zhou et al. (1998)and characterized by the AFLP PCR test developed by Branca et al. (2011). For 13 individuals sampled, *Wolbachia* was amplified but the strain could not be correctly identified.

2.7. Population genetic structure analysis

The genetic diversity of individuals (microsatellite data) was represented by multi-dimensional scaling according to their category or recovery site (MDS) (Fig. 3). In addition, the microsatellite data were analyzed using GENECLASS (INRA/CIRAD) and STRUCTURE (Pritchard et al., 2000; Hubisz et al., 2009) softwares. The post release samples recovered from maize or wild host plants were assigned to either the endemic population identified before the release, or to the introduced parental Kenyan strains.

3. Results

3.1. Recovery of Cotesia sesamiae from maize and wild host plants and GENECLASS assignation

The distribution of *C. sesamiae* on the different plant and stemborer hosts before and after release of Kenyan populations is presented in Table 2. Before its release, *C. sesamiae* was obtained only from noctuids

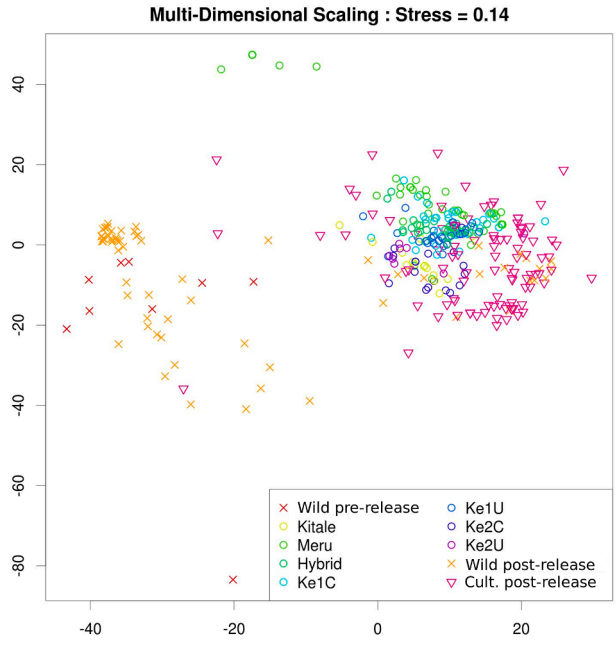

Fig. 3. Multidimensional scaling representation of two main axes of microsatellite genetic variation among wild pre-release (collected on wild host plants before releases of Kenyan *C. sesamiae*), introduced (Kitale, Meru, Hybrid : Kitale × Meru, Ke1: Meru × Kitale × Kakamega, Ke2: Meru × Kitale × Kakamega × Muranga × Mombasa × Tana River. C: Cured from *Wolbachia*. U: Uncured from *Wolbachia*. × : crossing), Wild Post-Release (collected on Wild host plants after Kenyan strains releases), Wild Post-Release (collected on cultivated host plants after releases).

stemborers feeding on wild host plants and never from *B. fusca* on maize, whereas after its release, of a total of 192 *C. sesamiae* cocoon masses, 96 were collected on maize and 96 on wild host plants. On maize, the stemborer species composition collected from 2007 to 2013 and the percentage of infestation by *C. sesamiae* are shown in Table 3. *Busseola fusca* was by far the most abundant stemboring species on maize, in both highland and forest regions, followed by *Eldana saccharina* (Walker) and *S. calamistis*. Parasitism on maize stemborers varied between 0 and 1.0 % with an average of 0.15% (Table 3). Among the 96 parasitoid samples collected on maize 45 were found in fields around the released sites and 51 in the vicinity of the parasitoid rearing unit in Yaoundé.

3.2. Genetic structure of introduced and endemic Cotesia sesamiae populations in wild and cultivated compartments

Individual females from 395 *C. sesamiae* cocoon masses were genotyped using microsatellite markers; 192 cocoon masses were from the seven introduced Kenyan strains released in Cameroon, 9 were from wild host plants before the releases of the Kenyan strains, 98 were collected from maize post-release, and 96 from wild host plants post-release. The representation of genetic distance by multi-dimensional scaling (MDS) is shown in Fig. 3. Eighty-six percent of the variation is explained by the two axes of the model presented in Fig. 3. We observed three genetic groups. A first "wild" group included the samples collected on wild host plants, namely all the 9 samples collected before release of the Kenyan strains, with 80 of the 96 genotyped insects collected on wild host plants post-release, and 3 of the 97 genotyped insects collected from maize post-release. A second "cultivated" group on the right of the first axis contained 185 of the 192 genotyped insects from the Kenyan released strains, 95 of the 98 genotyped insects collected from maize post-release, and 16 of the 96 insects collected on wild host plants post-release. Finally, a third group on the top of the second axis contained four insects from the released "Hy" Kenyan strain. The percentage of

Table 3
Number of stem- and ear- borers larvae and *Cotesia sesamiae* cocoon masses emerging on maize samples in the forest zone and high altitudes of Cameroon between 2007 and 2013. All *C. sesamiae* emerged from *B. fusca larvae.*

Ecozone	Year	# Fields	# Maize plants	# stem borers	% *B. fusca*	% *S. calamistis*	% *E. saccharina*	% Others	# *C. sesamiae*	% C. sesamiae
Forest	2007	28	1120	1309	55.23	1.07	42.40	1.30	13	0.99
	2008	81	3240	1663	90.68	5.35	3.91	0.06	11	0.66
	2009	80	3200	4173	97.84	0.41	1.75	0.00	4	0.10
	2010	64	2560	4028	99.38	0.00	0.62	0.00	40	0.99
	2011	182	7280	11,924	99.97	0.02	0.01	0.00	12	0.10
	2013	186	2232	3802	100.00	0.00	0.00	0.00	1	0.03
Total forest		**621**	**19,632**	**26,899**	**96.80**	**0.46**	**2.67**	**0.00**	**81**	**0.30**
Highlands	2007	26	1040	197	98.48	0	0	1.52	1	0.51
	2008	56	2240	1328	99.77	0	0	0.23	11	0.83
	2009	416	16,640	30,792	99.95	0.02	0	0.02	0	0.00
	2010	104	4160	4727	100	0	0	0	0	0.00
	2013	208	2496	3814	100	0	0	0	3	0.08
Total highlands		**810**	**26,576**	**40,858**	**99.95**	**0.00**	**0.0001**	**0.0003**	**15**	**0.04**
Total		**1431**	**46,208**	**67,757**	**98.70**	**0.0019**	**0.0106**	**0.0005**	**96**	**0.14**

"migrants" i.e. genetically from Kenya but observed on wild plants along with endemics (first group), or genetically from Cameroon but observed for the first time in cultivated fields (second group) was 16/98 = 16% for the first group and 3/96 = 3% for the second group.

The GENECLASS analysis provided similar percentages of "migrants". Of the 96 specimens collected post-release from wild habitats, 75 could be assigned to the endemic populations and 21 to the introduced populations (21% for the first group). Of the 96 specimens analyzed and recovered from maize post-release, 92 could be assigned to "migrants" and 5 to endemics (5% for the second group). According to GENECLASS, the number of "migrants" were 21 of 96 (22%) in wild habitats and 5 of 92 (5%) in cultivated habitats. If we consider only the individual with probability above 95% of being migrants, these values dropped to 19 (20%) and 4 (4%), respectively.

Structure analysis performed on all individuals, pre-release, introduced and post-release led to an optimal number of cluster based on Evanno's method of K = 2 (Suppl. Fig. 1, Evanno et al., 2005). It showed the same results as GENECLASS with 80.2% assignation to the same cluster of individuals collected post-release in wild habitats and individuals collected pre-release wild plant insects (19.8% of "migrants" in the first group) and 96.6% assignation to the same cluster of individuals collected post-release on maize and individuals from introduced strains (3.4% of "migrants" in the second group) (Table 5).

Wolbachia infection was detected in 84% of the 194 post-release samples. *Wolbachia* strain status, endemic or introduced matched by more than 95% that of the microsatellite markers. Sixty-one individuals were infected by endemic *Wolbachia*, of which 58 could be assigned to endemic microsatellite populations (95.1%). Eighty-four individuals were infected by introduced *Wolbachia*, of which 81 could be assigned to the introduced microsatellite population (96.4%) (Table 4).

Table 5
STRUCTURE assignation of the different populations: Prerelease (Wild plants), introduced (Me: Meru, Ki: Kitale, Hy: Hybrid Me × Ki, Ke1: Kenya 1 strain, Ke2: Kenya 2 strain, U: Uncured from Wolbachia, C: cured from Wolbachia), and recovered in wild (Post-release (Wild plants)) and cultivated (Post-release (Cultivated plants)) compartments after introduction. #Ind.: number of individuals. The model was admixed. Best number of cluster was 2 (S1 and S2 cluster).

	S1	S2	# Ind.
Pre-release (wild plants)	0.023	0.977	9
Me	0.998	0.002	10
Ki	0.989	0.011	17
Hy	0.998	0.002	15
Ke1U	0.998	0.002	43
Ke1C	0.787	0.213	47
Ke2U	0.974	0.026	30
Ke2C	0.987	0.013	30
Post-release (Wild plants)	0.198	0.802	96
Post-release (Cultivated plants)	0.966	0.034	98

4. Discussion

Results of the surveys show that the three specific objectives of the BC project were met: (1) the introduced *C. sesamiae* strains genetically designed to attack the major Cameroonian crop pest *B. fusca* were successfully recovered from *B. fusca* on crops; (2): the introduced insects were also recovered from wild hosts plants where 16% (MDS) to 21% (GENECLASS) of the insects collected were assigned to introduced *C. sesamiae*; (3): although endemic and introduced insects co-occur on wild host plants, the introduced *Wolbachia* remained associated at more

Table 4
Geneclass Assignation of *Cotesia sesamiae* individuals collected post release to endemic or introduced pouplations based on *Wolbachia* and nuclear microsatellite (SSR) compartments. Nuclear microsatellite asignement (in lines) was performed with the Geneclass software assignment (P > 0.5). *Wolbachia* strain asignment (in columns) was based on bacterial sequence. F|H: number of insects collected in Forest zone|Highlands (see Fig. 2). Underlined are the number of insects exhibiting SSR and *Wolbachia* of distinct origin.

			Wolbachia				Total
			Endemics F|H	Introduced F|H	Unknown F|H	Uninfested F|H	
Wild hosts	**SSR**	**Endemics**	**52**	**0**	**8**	**8**	**68**
			50|00	0|0	1|1	7|1	58|02
		Introduced	**3**	**13**	**4**	**1**	**19**
			3|0	13|00	1|2	0|1	17|03
Cultivated hosts		**Endemics**	**0**	**4**	**1**	**0**	**5**
			0|0	3|1	0|1	0|0	03|02
		Introduced	**0**	**84**	**0**	**9**	**93**
			0|0	75|09	0|0	8|1	83|10
		Total	**58**	**101**	**13**	**18**	
			53|00	91|10	2|4	15|03	164|17

than 95% with the introduced nuclear genome and the endemic *Wolbachia* remained associated at more than 95% with the endemic nuclear genome.

Almost all the populations recovered from maize after the biological control introductions could be assigned to the genetic cluster of the Kenyan strains released indicating that they established on maize in Cameroon. In contrast to the situation in high altitudes zones, the Kenyan populations successfully survived the dry season in the Inland Valley forest zone and were still recovered five years after the releases, probably because of the presence of continuous maize cropping (Thenkabail and Nolte, 1996; 1998; Ndemah, 1999; Almekinders and Hardon 2006) that have been shown to be reservoirs for non-diapausing *B. fusca* and *S. calamistis* and their natural enemies during the off-season (Chabi-Olaye et al., 2006). These fields along with wild grass habitats thus help perennating *C. sesamiae* during the off-season, when *B. fusca* in up-land fields are in diapause as larvae. Likewise, Chabi-Olaye et al. (2006) showed that during the dry season inland valleys harbour the scelionid egg parasitoids *Telenomus* spp., which move to upland maize fields at the beginning of the succeeding rainy season. Similarly in East Africa, cultivated and wild sorghum harbour non diapausing *B. fusca* during the dry season as well as several *Sesamia* spp. that perennate the parasitoid during the off season (Mailafiya et al., 2010). The presence of introduced *C. sesamiae* on wild host plant in the present study also underlines the role of this habitat during dry season.

In the high altitude, recoveries were made mostly during the same year as the releases in 2007 and 2008. Thereafter, only three cocoon masses were recovered in 2013. This scarcity of descendants from the introduced population may reflect the fact that the parasitoid may still be acclimatizing to its new environment, because it can be a long process as found for *C. flavipes* in East Africa (Midingoyi et al., 2016). Such a delay is common in biological control (Fauvergue et al., 2012). The reasons classically invoked are: (1) time for genetic adaptation to new environment, (2) *Allee* effect due to small initial population size, or (3) environmental stochasticity (Fauvergue et al., 2012). In our case, adaptation might be related to ability to survive in diapausing host. In Potchefstroom, South Africa, which is characterized by a five-month cool season, the population density of the first flight of *B. fusca* was largely dependent on the incidence of parasitism of diapausing larvae by *C. sesamiae* (Rensburg van and Walters, 1987). Similarly, Rensburg van and Walters (1987) observed high mortality of field-collected larvae caused by *C. sesamiae* in cold storage. Thus, it appears that *C. sesamiae* is able to survive during the non cropping season in diapausing *B. fusca* larvae. This was verified by B. Le Ru (pers. Observations), who obtained *C. sesamiae* from diapausing *B. fusca* larvae collected in Kitale, which is situated in the highland zone in Kenya. In the present study, the sample size might have been too small to allow detection of *C. sesamiae* in the highlands of Cameroon so soon after the first releases. Also, additional releases should be considered to help establishment and spread of the parasitoid in the highlands.

The results show that despite the co-occurrence of endemic and introduced populations on refuge host plants, the two populations did not interbreed at a significant rate. Five years post release there appeared to be a strong separation of the introduced genomes on one hand and that of the endemics on the other hand. *Wolbachia* is likely to contribute to this separation. Endemic *Wolbachia* were associated at more than 95% with endemic nuclear genes and introduced *Wolbachia* were associated at more than 95% with introduced nuclear genes. There is therefore some permeability of the *Wolbachia* compartments. This is consistent with the observation that cytoplasmic incompatibility is incomplete (Branca et al., 2019). Branca et al. (2019) also observed that cytoplasmic incompatibility was stronger for *A + Beast* (endemic) *Wolbachia* variants than for *Bwest* (introduced) variants. There should therefore be less gene flow from introduced to endemics than the reverse. On maize, endemic *Wolbachia* were not recovered while on wild host plants introduced *Wolbachia* were present. The genetic exchanges are therefore likely to occur in wild habitats first. Most of the introduced individuals found on wild host plants were also infected by introduced *Wolbachia* strains but there were also three introduced individuals with endemic *Wolbachia* recovered and five individuals not infected. This asymmetric and incomplete cytoplasmic incompatibilityI can be due to an effect of parasitoid genome (Bordenstein and Werren, 1998). A possible scenario is therefore introgression in wild habitats due to incomplete cytoplasmic incompatibility. Models predict that linkage disequilibrium between *Wolbachia* and nuclear genes would break down quickly if other forces do not maintain isolation (Telschow et al., 2002). Branca et al. (2019) confirmed that host species contributed to genetic differentiation even more than *Wolbachia* on an African scale. Since *C. sesamiae* mostly reproduce immediately after emergence on host larvae, the role of host in genetic differentiation may be effective, even for populations present in the same habitat (Branca et al., 2011). The reduction of genetic exchanges between the introduced insects and the endemic compartments is therefore likely to be reinforced by the difference in insect host species associated to these compartments.

The key for conciliating biological control efficiency and safety in our case was the use of scientific knowledge on population biology of the biological control agents and their wild relatives and molecular genetics tools to allow refuge habitat to contribute to population dynamics without negative interaction between introduced and endemic populations. It was shown that introduced populations need wild habitats but must not interbreed with endemics. It is necessary to conduct long term follow-up studies in both host plant compartments to confirm this hypothesis of *Wolbachia* driven genetic protection of endemic populations from introduced conspecific biological control agents. The confirmation of such hypothesis, namely that reproductive isolation by *Wolbachia* would play a role in keeping local adaptations of endemic insects toward their natural hosts and may preserve their native ecological functions, would be a further success of the project. This would require a genetic marker of adaptation. The CrV1 gene would be a good candidate because it has an allele that is almost endemic to Cameroon and associated with the wild hosts *Poeonoma serrata* (Hmps.) (Lep.: Noctuidae) (Branca et al., 2011). The CrV1 virulence gene could also be used as a diagnostic marker of adaptation to *B. fusca* by harbouring alleles linked to parasitoid success on this host (Dupas et al., 2008). The gene evolves through natural selection and is genetically linked to factors of suppression of local host resistance (Dupas et al., 2008; Jancek et al., 2013). Yet recent genomic studies suggest CrV1 is not the causal factor but just a marker due to the co-segregation of the virus in the parasitoid genome (Gauthier et al., 2020). Diagnostic PCR tests will have to be developed in order to follow up populations as well as to bring in new introductions by confirming the cohesion between *Wolbachia*, microsatellites and polydnavirus markers.

5. Data statement

The molecular data is given as supplementary data (SupplementaryData.xlsx).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2020.104478.

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