Determinants of genetic structure of the Sub-Saharan parasitic wasp *Cotesia sesamiae*

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Abstract

Parasitoid life style represents one of the most diversified life history strategies on earth. There are however very few studies on the variables associated with intraspecific diversity of parasitoid insects, especially regarding the relationship with spatial, biotic and abiotic ecological factors. *Cotesia sesamiae* is a Sub-Saharan stenophagous parasitic wasp that parasitizes several African stemborer species with variable developmental success. The different host-specialized populations are infected with different strains of *Wolbachia*, an endosymbiotic bacterium widespread in arthropods that is known for impacting life history traits notably reproduction, and consequently species distribution. In this study, first we analyzed the genetic structure of *C. sesamiae* across Sub-Saharan Africa, using 8 microsatellite markers, and 3 clustering software. We identified five major population clusters across Sub-Saharan Africa, which probably originated in East African Rift region and expanded throughout Africa in relation to host genus and abiotic factors such as climatic

classifications. Using laboratory lines, we estimated the incompatibility between the different strains of *Wolbachia* infecting *C. sesamiae*. We observed an incompatibility between *Wolbachia* strains was asymmetric; expressed in one direction only. Based on these results, we assessed the relationships between direction of gene flow and *Wolbachia* infections in the genetic clusters. We found that *Wolbachia*-induced reproductive incompatibility was less influential than host specialization in the genetic structure. Both *Wolbachia* and host were more influential than geography and current climatic conditions. These results are discussed in the context of African biogeography, and co-evolution between *Wolbachia*, virus parasitoid and host, in the perspective of improving biological control efficiency through a better knowledge of the biodiversity of biological control agents.

KEYWORDS: *Cotesia sesamiae*, parasitoid wasp, *Wolbachia*, genetic structure, host specialization.

Introduction

Understanding the extraordinary biodiversity of insects requires both analyzing large scale beta diversity patterns (Heino *et al.* 2015) and unraveling mechanisms of genetic differentiation among populations including geographic, abiotic or biotic interactions (Roderick 1996). Parasitoid wasps are one of the most diverse groups of insects (Grimaldi 2005). Coevolutionary interactions are likely major diversifying forces in host–parasitoid systems due to the strength of reciprocal selection pressures (Van Valen 1973; Henry *et al.* 2008). As strong insect antagonists, they are the most used agents for biological control programs, which provide one of the best alternatives to chemical control of insect pests (Harvey 2011). There are theoretical expectations that host parasitoid coevolution generates diversity because several traits related to host specificity, such as specific virulence and host recognition, are mechanistically linked to reproductive isolation, especially when the parasitoid mates on the host just after emergence (Dupas *et al.* 2008; Hoskin & Higgie 2010). Other biotic interactions, particularly those involving microorganisms affecting reproduction such as *Wolbachia* sp., are expected to drive diversification of parasitoids (Bordenstein *et al.* 2001; Branca *et al.* 2009). To distinguish between the different ecological factors responsible for population structure, a combination of, on the one hand, laboratory data on reproductive incompatibility and, on the other hand, field data on the geographic structure of ecological drivers and population differentiation are needed.

Cotesia sesamiae Cameron (Hymenoptera: Braconidae) is a parasitoid wasp widespread in Sub-Saharan Africa that has been used in biological control for controlling *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), a major stemborer pest of maize and sorghum crops (Kfir 1995; Kfir *et al.* 2002). *Cotesia sesamiae* is a stenophagous parasitoid that successfully parasitizes diverse host species (Ngi-Song *et al.* 1995; Branca *et al.* 2011). However, a variation in parasitism success on different hosts has been shown among

68 populations of parasitoids (Mochiah *et al.* 2002a; Gitau *et al.* 2010). In contrast to the *C.*
69 *sesamiae* population from Mombasa - coastal Kenya (avirulent towards *B. fusca*), the *C.*
70 *sesamiae* population from Kitale – inland Kenya (virulent towards *B. fusca*) is able to
71 develop in *B. fusca*, but both develop in *Sesamia calamistis* Hampson (Lepidoptera:
72 Noctuidae), the main host of *C. sesamiae* in coastal Kenya (Ngi-Song *et al*. 1995). These
73 differences in host acceptance and development have been linked to the observed
74 polymorphism of a candidate gene, CrV1, located on the bracovirus locus (Dupas *et al.* 2008;
75 Gitau *et al.* 2007; Branca *et al.* 2011). Bracoviruses are symbiotic polydnaviruses integrated
76 to the genome of braconid wasps, contributing to their adaptive radiations (Whitfield 2002;
77 Dupuy *et al.* 2006). The viruses constitute the major components of the calyx fluid of the
78 wasp and are expressed in parasitoid host cells, regulating its physiology, development and
79 immunology (Beckage 1998). In particular, the CrV1 gene, has been shown to contribute to
80 immune suppression by active de-structuration of the cytoskeleton of host immune cells
81 (Asgari *et al.* 1997). A comparative genomics study of the virus between *Cotesia* species and
82 *C. sesamiae* populations, virulent and avirulent against *B. fusca*, showed patterns suggesting
83 important role for positive selection, gene duplication and recombination among viral genes
84 in the adaptive diversification process (Jancek *et al.* 2013). Whilst host resistance puts likely
85 a strong selective pressure on local adaptation of the wasp, other ecological and geographic
86 factors must be considered and analyzed for the development of scenario of *C. sesamiae*
87 response to environmental changes. Climatic differences or geographical barriers might
88 weaken the capacity of some *C. sesamiae* populations to colonize areas where the most
89 prevalent host is suitable for parasitoid larval development, even if parasitic wasps have been
90 shown to disperse quite efficiently, sometimes beyond the capacity of their associated host
91 (Antolin & Strong 1987; Ode *et al.* 1998; Van Nouhuys & Hanski 2002; Assefa *et al*. 2008,
92 Santos & Quicke 2011). Other factors such as *Wolbachia* might act as a barrier to gene flow

93 through reproductive incompatibility (Werren 1997; Jaenike *et al.* 2006), which can be
94 especially problematic in the context of biological invasions by preventing crosses between
95 ecological or geographic populations along the range expanded area of the invasive pest host.
96 *Wolbachia* is a widespread bacterium infecting the majority of insect species that can induce
97 reproductive incompatibilities (Werren 1997; Hilgenboecker *et al.* 2008). Several *Wolbachia*
98 strains have been identified in *C. sesamiae* expressing cytoplasmic incompatibilities (CI)
99 between populations of parasitoids (Mochiah *et al.* 2002b). The different populations of *C.*
100 *sesamiae*, virulent and avirulent against *B. fusca*, are infected with different strains of
101 *Wolbachia* (Branca *et al.* 2011). Reproductive isolation can prevent adapted parasitoid
102 populations to expand across their host range, a phenomenon that could be particularly
103 relevant in biological control programs. In this study, our objective is to analyze the relative
104 importance of neutral geographic factors and major selective forces, biotic (*i.e.* host species
105 and *Wolbachia* strain), abiotic (*i.e.* climate) shaping the genetic structure of the parasitoid
106 *Cotesia sesamiae* across Sub-Saharan Africa. First, the genetic structure was assessed using 8
107 microsatellites markers with several genetic clustering approaches, each using different
108 pertinent hypotheses in an effort to reach the broadest picture of the structure. Second, we
109 tested the cross incompatibility between differentially *Wolbachia*-infected *C. sesamiae*
110 populations to infer their potential influence on limiting gene flow. Third, we estimated the
111 amount and direction of gene flow in between genetic clusters of selected *C. sesamiae*
112 populations to see if *Wolbachia* infection can affect the parasitoid metapopulation dynamics.
113 Finally, we interpreted geographic patterns of *C. sesamiae* genetic structure in the context of
114 African climate, *Wolbachia* infection and host occurrence.
115

116 **Material and methods**

117

118 *Insects field collection*

119 Infected stemborer larvae were collected in 142 localities of 9 sub-Saharan African
120 countries. GPS positions were recorded at each locality. Stemborer larvae were identified
121 using a larval picture library (corresponding to adult moth identifications), and according to
122 the host plant, as most stemborers are host plant specific (Le Ru *et al.* 2006). Larvae collected
123 from the field were reared on an artificial diet (Onyango & Ochieng'-Odero 1994) until
124 pupation or emergence of parasitoid larvae. After the emergence of cocoons, adult parasitoids
125 were kept in absolute ethanol. Morphological identification of parasitoids was based on
126 genitalia shape following the method of Kimani-Njogu *et al.* (1997). Total genomic DNA of
127 one female per progeny was extracted using the DNeasy Tissue Kit (QIAGEN). If only male
128 were present then analyses were performed on one male. Because wasps are haplodiploids,
129 the haploid genotype of males was converted to homozygous diploids for analyses to avoid
130 discarding data. This should not bias the results because of a very low level of heterozygosity
131 due to very high inbreeding. Total genotyped individuals were 590 females and 47 males
132 discarding individuals with too many missing genotypes (more than 2 over 8 loci).

133

134

135 *Insects rearing*

136 For crossing experiments, females of both virulent and avirulent *C. sesamiae* strains
137 against *B. fusca* were obtained from laboratory-reared colonies. The virulent, thereafter
138 named Kitale (Kit) *C. sesamiae* strain was obtained from *B. fusca* larvae collected from
139 maize fields in Kitale, Western Kenya, in 2006, while the avirulent *C. sesamiae* strain
140 thereafter named Mombasa (Mbsa), was obtained from *S. calamistis* larvae collected from

maize fields in coastal Kenya in 2007. The two lines have different *Wolbachia* infection status: Kitale line is infected with *Wolbachia* WCsesB1 strain while Mombasa line is infected with two strains of *Wolbachia* WCsesA and WCsesB2 (Table 1). Twice a year, both colonies were rejuvenated by field collected parasitoids. The wasps of both strains were continuously reared on larvae of *S. calamistis* as previously described Overholt *et al.* (1994). Parasitoid cocoons were kept in Perspex cages (30 x 30 x 30 cm) until emergence.

Adults were fed a 20% honey–water solution imbibed in a cotton wool pad and kept under artificial light for 24 h to mate. In all experiments, only 1-day-old females, putatively mated and unexperienced to oviposition were used. The experiments were carried out at 25 ± 2 °C, 50– 80% RH, and a 12:12 h (L:D) photoperiod.

The stemborer species, *B. fusca* and *S. calamistis*, were continuously reared on artificial diet as previously Onyango & Ochieng'-Odero (1994). For each species, three times a year, several stemborer larvae were added to rejuvenate the colonies. Fourth larval instars were introduced into jars (10 x 20 cm), each containing pieces of maize stem, and left for 48 h to feed and produce frass to facilitate host acceptance by the parasitoid wasps for parasitism experiments. Thereafter, the larvae were used in the experiments.

Genetic markers sequencing and genotyping

Eleven previously developed microsatellites markers (Jensen *et al.* 2002; Abercrombie *et al.* 2009) were amplified and fragment size determined. Amplifications were performed in 10 µL with approximately 5 ng of genomic DNA, 1 × HotStarTaq PCR buffer, 2 µL Q-Solution 5× (QIAGEN), 1.6 mM of dGTC, dTTP and dCTP, 50 µM dATP, 5 pM of each primer, 0.25 U Taq polymerase (HotStarTaq, QIAGEN) and 0.01 U of [33P]-dATP. The 'touchdown' PCR (Mellersh & Sampson 1993) was used as follows: initial activation step at 95°C for 15 min, 18 cycles at 94°C for 30 s, 60 to 51°C for 30 s (-0.5°C/cycle), 72°C for 30

166 s, 29 cycles at 94°C for 30 s, 54°C for 30 s, 72°C for 30 s and a final elongation step at 72°C
167 for 10 min. Results were visualized using an ABI 310 and a AB3130 sequencer with
168 fluorescent size standard (GeneScan 600 Liz, Applied Biosystem). Amplifications were made
169 following conditions previously described using fluorescent labeling (Pet, Vic, Ned or 6Fam)
170 of the forward primer.

171 Peaks identifying fragment sizes were assessed using GeneMapper 4.0 Software.
172 Locus B1.42 presented peaks difficult to analyze with multiple bumps preventing any
173 accurate measure of fragment size and was thereby discarded. Loci B1.155 and B5.126 were
174 also not considered in the analyses because they presented a high percentage of missing
175 genotypes (respectively 14.6% and 27.0%) probably reflecting the occurrence of null alleles.
176 Eight loci were then genotyped per individual.

177 *Wolbachia* infection status was checked using the protocol developed in Branca *et al.*
178 (2011).

179

180 *Cross-mating experiment*

181 To obtain *Wolbachia*-free parasitoids colonies (named cured lines), the gravid females
182 of each aforementioned parasitoid line were reared on larvae of *S. calamistis* previously fed
183 on artificial diet Onyango & Ochieng'-Odero (1994), enriched with 2000 mg/L rifampicine
184 (Dedeine *et al.* 2001). This process was repeated for three generations of female wasps to
185 create cured colonies of Mombasa (Mbsa) and Kitale (Kit) *C. sesamiae*.

186 Cross experiment tests were conducted between both Mbsa and Kit *C. sesamiae* lines
187 to assess the mating incompatibilities due to the presence of different *Wolbachia* types.
188 Individual parasitoids were allowed to emerge singly by separating single cocoon from each
189 cocoons mass. Individual male and female parasitoids from each colony (i.e. Kit *C. sesamiae*
190 cured and uncured as well as Mbsa cured and uncured) were used for cross-mating

191 experiments. Sixteen possible cross-mating combinations were investigated (Table 4). Each
192 cross-mating combination was repeated at least 20 times.

193 After mating, females were presented 4th instar larvae of *S. calamistis* for oviposition
194 using the method of Overholt *et al.* (1994). Thereafter, the larvae were reared and observed
195 daily for mortality or parasitoid emergence. The developmental time of the progeny (egg to
196 adult), the brood size, the sex ratio and the mortality outside and inside the host were
197 recorded.

198 The presence of *Wolbachia* infections, in all *C. sesamiae* populations used in the
199 cross-mating experiments, was tested using PCR techniques on *ftsZ* and *wsp* genes as
200 described by Ngi-Song & Mochiah (2001). DNA was extracted from about 50 individuals (a
201 mixture of males and females) from each population previously stored in 99% ethanol.

202 To test the effect of mating direction on each reproductive trait, a non-parametric
203 Kruskal-Wallis test was applied with crosses as factor. ANOVA was not used because none
204 of the data were normally distributed and had homoscedastic variance. Following Kruskal-
205 Wallis test, a pairwise Wilcoxon's rank sum test was conducted with false discovery rate
206 (FDR) correction for multiple testing. Data were split into four groups for statistical analyses:
207 crosses between Kit wasps, crosses between Mbsa wasps and crosses between populations in
208 both directions. For all crosses, CI is expected between infected males and uninfected or
209 differentially infected females. CI should lead to a reduction in female production either by
210 female mortality (FM phenotype, diminution of the size of the progeny and the number of
211 females) or male development (MD phenotype, only diminution of the proportion of females)
212 (Vavre *et al.* 2000).

213 Statistical analyses for *Wolbachia* crosses experiments were performed in R 3.2 (R
214 Core Team 2013).

215

Genetic structure inference

To infer population structure from genetic data we used three different Bayesian methods for population partitioning: INSTRUCT, based on Hardy-Weinberg equilibrium with inbreeding (Gao *et al.* 2007), TESS3, taking into account spatial autocorrelation based on tessellation (Caye *et al.* 2016) and DAPC, a statistical partitioning method based on PCA (Jombart & Ahmed 2011). First, Instruct software was used with the Adaptive Independence Sample algorithm using inbreeding coefficient at population level as a prior model (mode 4, option v) (Gao *et al.* 2007), since *C. sesamiae* is known to have a highly inbred reproductive system (Ullyett 1935; Arakaki & Ganaha 1986). The number of clusters corresponding to the strongest genetic structure was determined using the method of Evanno *et al.* (2005). Each inference had a total number of iterations of 200,000 with a burn-in period of 100,000 iterations. Other parameters were kept as default value except the significance level of the posterior distribution of parameters, which was set to 0.95. The posterior probability of assignation of each individual was re-calculated over 10 MCMC runs using the CLUMPP software (Jakobsson & Rosenberg 2007) with greedy algorithm and 10,000 random permutations. Second, TESS3 was run using admixture with the BYM model (Durand et al. 2009). To identify the strongest structure, the model was run with K ranging from 2 to 9 using 100,000 sweeps with a 10,000 burning period. Degree of trend was assessed by running the algorithm with a varying value from 0 to 3 by 0.5 steps. The degree of trend showing the best DIC was kept. Genetic structure was then assessed for K=5, the best K, and T=1.5, the best degree of trend, with MCMC chain run for 1,000,000 sweeps with a 100,000 burn-in period. Third, we used a PCA-type approach with DAPC in R package *adegenet* (Jombart & Ahmed 2011) which is hypothesis-free since it just clusters individuals to maximize the explained genetic variance within the data.

The influence of various factors on the genetic variance was described using multiple

241 correspondence analysis (MCA, package FactoMineR), and assessed using the *adonis*
242 function in vegan R package (Oksanen *et al.* 2013). This corresponds to an extension of
243 AMOVA (Excoffier *et al.* 1992) for crossed factors and in a non-hierarchical pattern
244 (McArdle & Anderson 2001). The factors considered were: host genus, the *Wolbachia*
245 infection status, spatial cluster of samples and the Köppen-Geiger climate type (Kottek *et al.*
246 2006). As the sampling was not done randomly across Sub-Saharan Africa, we tested the
247 effect of spatial structuration by defining spatial cluster grouping localities close to each
248 other. The spatial cluster of samples was obtained with hierarchical clustering from latitude
249 and longitude data (*Mclust* function) (Fraley & Raftery 2002; Fraley *et al.* 2012). Genetic
250 distance between individual were generated using Smouse Peakall's formula (Smouse &
251 Peakall 1999) in GenoDive (Meirmans & Van Tienderen 2004).

252 A Bayesian analysis of population sizes and reciprocal migration rates between the
253 consensus genetic clusters obtained from partitioning methods was performed using software
254 Migrate (Beerli & Palczewski 2010). Migrate-n software version 3.6.6 was run using the
255 microsatellite model set to Brownian motion and the gene flow model to asymmetric. Since
256 asymmetric gene flows can only be estimated pairwise, we run independently the software
257 for each pairwise cluster comparison. Prior distributions of θ and M were chosen to get
258 posterior distributions that are not truncated. Five chains of different heat from 1 to 10 were
259 run for 500,000 generations with a burn-in period of 10,000.

260

261 **Results**

262 *Genetic structure*

263 The three clustering methods, Instruct, DAPC and TESS3 used in this study gave
264 similar results regarding the population structure of *C. sesamiae* populations. For each
265 method, the best fit was observed for five clusters (maximum delta-K for Instruct, Figure S1,

266 diffNgroups criterion for DAPC and Deviance Information Criterion for TESS3). Regarding
267 the structuration in relation to the host species, cluster 1 of all three methods was found
268 exclusively on *Sesamia nonagrioides* (Figure 1, in red), clusters 2 and 3 were found mainly
269 on *Busseola ssp.* (Figure 1, in yellow and green, respectively), cluster 4 on *Sesamia* and *Chilo*
270 *spp.* (Figure 1, in blue) and finally cluster 5 was recovered from five host genera (Figure 1, in
271 purple). Geographically, the three methods provided similar picture of genetic structure with
272 some difference in admixture proportion. Cluster 1 population was scattered in between
273 central Ethiopia, western Kenya and Northern Tanzania, and even Cameroon (Figure 2 in
274 red). This corresponded to the population found on *S. nonagrioides*. One discordance
275 appeared with the DAPC method, which failed to assign one individual from Arusha
276 (Tanzania) into Cluster 1. Cluster 2 extended from Eritrea to Western Kenya in Instruct, but
277 was restricted to Western Kenya in TESS3 and DAPC (Figure 2, yellow). Conversely, cluster
278 3 was only present in Western Kenya and central Tanzania in the three methods but extended
279 to Eritrea in TESS3 and DAPC (Figure 2, green). Cluster 4 extended from South Africa to
280 East Kenya and Rwanda in the three methods (Figure 2, blue). In Instruct and TESS3
281 analyses, a very high posterior probability of cluster 4 was also observed further west in the
282 coast of Congo-Brazzaville. Cluster 5 extended from Tanzania to Cameroon in all three
283 methods but was found much more spread in DAPC analysis, until South-Africa, and to a
284 lesser extent in Instruct (Figure 2, purple). Overall, there seem to be a clear delimitation
285 between cluster 2 and 3, which extend form Tanzania to Eritrea, and cluster 4 and 5, which
286 were found from Cameroon to South Africa. Delimitation within these two groups of two
287 clusters seemed to be shallower and influenced by the method used.
288
289 Wolbachia *strains distributions*
290 A rather good concordance was observed between genetic structure at microsatellites level

and *Wolbachia* strain distributions (Figure 3). Cluster 1 was associated exclusively with the *Wolbachia* wCsesA strain, cluster 2 and 3 with wCsesB1, cluster 4 and 5, with the bi-infection wCsesA/wCsesB2.

Relative influence of biotic and abiotic factors

The individuals belonging to the cluster found exclusively on *Sesamia nonagrioides* were interpreted as a distinct species by Kaiser *et al.* (2015, 2017), based on eco-phylogeny analyses and cross-mating experiments, and corresponding to a host and plant-host driven ecological speciation event. As it has now been described as the species *Cotesia typhae* (Kaiser et al. 2017), it was not considered in these analyses to prevent an overestimation of host effect. Multiple correspondence analysis (Figure 4) suggested the presence of structure in relation to all the factors considered (spatial cluster, Köppen-Geiger climate classification, *Wolbachia* infection status and host genus). The full models tested with *adonis* function (Table 2) confirmed that all neutral (geography) and selective forces, abiotic (climate type and geography) and biotic (host genus, *Wolbachia* infection), contribute significantly to the genetic variance of the microsatellite genotypes. Because the *adonis* method tests factors sequentially, it is important to consider each factor as either first term or marginal (last) term to see the extent of the effect. When added first in the sequence of factors in the *adonis* function, the biotic factors had higher R^2 than the abiotic factors (0.43 and 0.38 for *Wolbachia* and host genus, respectively and 0.28 and 0.21 for Köppen-Geiger Climate type, and localization, respectively) (Table 3). In addition, all the factors had significant marginal effects (Table 3). Pairwise interactions between factors were weak (R^2<0.04), but significant for all the possible interactions (Table 2). None of the tripartite interactions was significant.

Wolbachia *crosses experiment*

316 For crosses within each population, the brood size dropped in crosses involving
317 infected males and cured females (i.e. Cs Kit x Cs Kit-cured and Cs Mbsa x Cs Mbsa-cured)
318 from 34-36 to 23 for Kitale population and from 32-42 to 21 for Mombasa population (Table
319 4). Although for these both potentially incompatible crosses the sex ratio (or %females)
320 decreased significantly for Kitale population and no significant change was detected for
321 Mbsa population, the overall number of females was reduced in these both crosses from 45-
322 62% to 44% and from 57-64% to 55% for Kitale and Mbsa population, respectively. No
323 significant changes in the developmental time and in the mortalities outside and inside the
324 host through dissection were detected between these incompatible crosses and the other
325 crosses.

326 To the contrary, in crosses potentially showing bidirectional CI, i.e. crosses involving
327 individuals from different populations and infected with different *Wolbachia* strains (i.e. Cs
328 Kit x Cs Mbsa and Cs Mbsa x Cs Kit), we only found a significant decrease of the percentage
329 of female from 47-67% to 11-0% in the cross involving Mbsa males and Kit females (Table
330 4). In this cross, almost no females were recovered despite a normal overall progeny size,
331 suggesting a complete incompatibility with pure male development (MD) phenotype (Vavre
332 *et al.* 2000). By contrast, in the cross for Kit male (wCsesB1) with Mbsa female
333 (wCsesA/wCsesB2), CI expressed only when Mbsa females were cured and only partially
334 since females were recovered. No significant changes in the developmental time and in the
335 mortalities outside and inside the host through dissection were detected between these
336 incompatible crosses (i.e. Cs Kit x Cs Mbsa-cured, Cs Kit x Cs Mbsa, Cs Mbsa x Cs Kit-
337 cured and Cs Mbsa x Cs Kit) and the other crosses.

338

339 *Migration patterns*

340 For Bayesian analyses of pairwise migration rates, acceptance rate ranged between

0.20 and 0.56 with an effective MCMC sample size from ~500 to ~2700. Clusters defined by Instruct were used except Cluster 1 for the main reasons as exposed above. Mostly symmetric gene flow was found between Cluster 2 and 3, which are mainly infected with the same wCsesB1 *Wolbachia* strain (Figure 5); they were found mainly on *Busseola*, at least in one contact zone in Central Kenya (Figure 2). Otherwise, asymmetric gene flow between clusters were found. All the gene flow with cluster 5 were orientated toward Cluster 5. Gene flow between Cluster 4 (found mainly on *Sesamia* and *Chilo*) and Cluster 2 was the lowest despite the presence of a contact zone in Kenya (Figure 2). Kit population from the laboratory colony was assigned to Cluster 2 and Mbsa population from the laboratory rearing to cluster 5 as inferred in Instruct clustering (Table 1). Therefore, migration was more orientated from wCsesB1-infected population toward wCsesA/wCsesB2 bi-infected populations, mainly because of an asymmetric gene flow in that particular direction between Cluster 3 and Cluster 4.

Discussion

Geographic, ecological and biotic factors determining the genetic structure of **Cotesia sesamiae**

The five major clusters inferred by the three different genetic clustering methods, TESS, Instruct and DAPC exhibited very similar geographic partition. However, TESS3 and Instruct admixture models were more concordant. DAPC results differed by the many geographic areas assigned to just one cluster. The DAPC algorithm optimizes a model without admixture that assigns individuals and not portion of their genomes to clusters. It seeks linear combination of genetic variables that maximizes between clusters component of the genetic distance between individuals. Models without admixture are not robust to the

366 inclusion of admixed individual in the sample, reciprocally, models with admixture are less
367 able to detect barriers when admixture is limited (François *et al.* 2010). In the absence of
368 intrinsic biological reproductive barriers between the populations, we would expect the
369 admixture model is the best suited because the five clusters are all represented in Kenya and
370 Tanzania with a geographic continuum in both countries. However, the presence of
371 reproductive isolation mechanisms, may limit admixture in this continuum of populations.
372 Indeed, the results of Instruct non-spatial admixture model (Figure 2) shows that populations
373 maintained their integrity; admixture being limited to the hybridization zones despite the
374 ability of *C. sesamiae* to expand throughout Africa. We will discuss below the factors that
375 may limit admixture in this continuous environment in the light of our results on
376 experimental crosses, *Wolbachia* strains distribution, host ecological specialization, climate,
377 and on the biology of *C. sesamiae*.
378
379 There are at least three strains of *Wolbachia* infecting *C. sesamiae* populations across
380 Sub-Saharan Africa (Branca *et al.* 2011). We did not find bidirectional incompatibility
381 between populations as a result of different infection. Only individuals infected with wCsesA
382 and wCsesB2 strains showed incompatibility with cured or wCsesB1 infected Kit individual.
383 Infected individuals wCsesA/wCsesB2 were already found highly incompatible in a previous
384 study (Mochiah *et al.* 2002b), but incompatibility was not assessed for wCsesB1-infected
385 individuals. The results for *Wolbachia* crosses involving wCsesB1 infected males and cured
386 females does not present normal CI phenotype because there was no increase in male
387 proportion in the progeny; however, we observed a reduction in progeny size (males and
388 females) leading to a reduced number of females. This result is coherent with *Wolbachia*
389 invasion theory since *Wolbachia* fitness is linked to the fitness, which female progeny size is
390 a proxy, of *Wolbachia*-infected females relative to non-infected counterparts (Werren, 1997).

391 However, this means that there is an unknown mechanism leading to high mortality of male
392 eggs in incompatible crosses. Possibly, part of the male progeny includes in fact diploid
393 males that could be affected in incompatible crosses, as diploid males are common in *Cotesia*
394 wasps (Zhou *et al.* 2006; De Boer *et al.* 2007). A direct effect on development, not related to
395 fertilization, could be also considered. In a similar way, surprisingly, no strong
396 incompatibility was observed between Mbsa wCsesA/wCsesB2 cured female and Mbsa
397 infected males as no biased sex ratio was found in the progeny. However, as in the case of
398 Kit, a reduction in progeny size was observed which means that probably CI expresses
399 differently between individual of the same genetic background (Kit or Mbsa), than when
400 incompatible crosses occur between different genetic backgrounds. In the inter-population
401 crosses studied here, a MD phenotype is very likely, as male-biased sex ratio was not
402 associated to significant progeny size reduction. The consequence of this unidirectional
403 incompatibility will be asymmetric gene flow between differentially infected populations
404 (Jaenike *et al.* 2006; Telschow *et al.* 2006). Indeed, CI is an efficient mechanism for
405 *Wolbachia* to spread within populations by giving infected females a higher fitness. We
406 should therefore expect the spread of individuals infected with wCsesA/wCsesB2 across *C.*
407 *sesamiae* geographical range, reflected by higher migration rate from wCsesA/wCsesB2-
408 infected populations toward other populations. However, using microsatellite markers, we
409 observed conversely a lower migration rate from wCsesA/wCsesB2 -infected genetic clusters
410 toward the other clusters (Figure 5), except for the migration between cluster 4 and 2. This
411 unexpected result may be explained by local adaptation. Regions where *C. sesamiae* are
412 infected with wCsesA/wCsesB2 are indeed dominated by avirulent parasitoids and
413 susceptible hosts, whereas regions where *C. sesamiae* are infected with wCsesB1 are
414 dominated by virulent parasitoid attacking resistant host. Females migrating from bi-infected
415 to wCsesB1 regions are maladapted and killed by encapsulation, but females migrating from

wCsesB1 to regions with wCsesA/wCsesB2-infected individuals are able to develop on the host. Yet, males infected with wCsesB1 can reproduce with bi-infected females wCsesA/wCsesB2, which would allow some gene flow from wCsesB1 to wCsesA/wCsesB2. In conclusion, *Wolbachia* incompatibility favors the expansion of avirulent parasitoid wasp that are not capable to survive in some areas, and, in the opposite, the spread of virulent parasitoid is limited by area where parasitoid population are dominated by individuals infected with highly incompatible Wolbachia. This situation should lead to potentially stable contact zone between populations and current genetic structure.

To disentangle the effects of geography, *Wolbachia* infection, parasitoid host, and other ecological factors, a statistical model was optimized using *adonis* R function. The biotic and abiotic factors including geography analyzed in our statistical model explained more than 75% of the genetic variance. When looking at the factors most correlated to the genetic structure, our results are consistent with the hypothesis that ecology plays a significant role in reinforcing *C. sesamiae* population structure across evolutionary time. Indeed, *adonis* analysis showed that the strongest determinant of genetic variance was *Wolbachia* infection followed by the host species and the least contributing factors were localization and climate. An illustration of the dominant effect of the host is the particular status of the population represented by cluster 1, consistently collected on *Sesamia nonagrioides*. This population shows also higher Fst when compared to the other populations in every clustering method confirming it constitutes a new species as it has recently been proposed (Kaiser *et al.* 2015, 2017). Another population corresponding to cluster 5 expands from Cameroon to East-Africa and Uganda, trough Democratic Republic of Congo; this region corresponds to the great Equatorial forest of Africa, which is characterized by hot and wet climatic conditions. The cluster 4, located from Eastern Kenya to Mozambique along the Coast, is situated in a much drier area than cluster 5. This area is also important regarding host, since *B. fusca*,

441 characterized as a resistant host, is rare in those regions (Le Ru *et al.* 2006; Moolman *et al.*
442 2014). The cluster 2 and 3 are located both in North-Eastern Sub-Saharan Africa but their
443 positions differed according to clustering algorithm (i.e. West Kenya, Ethiopia and Eritrea).
444 In terms of climatic conditions, these regions are very similar but the observed clusters might
445 reflect two sympatric populations with recurrent gene flow as they are infected with the same
446 *Wolbachia* strains (Figure 3).

447 These *C. sesamiae* populations show some geographic similarities with the genetic
448 structure observed in the known resistant host *B. fusca* (Dupas *et al.* 2014), with five clusters
449 observed across Africa, and a strong structure observed in East African Rift Valleys regions,
450 contrasting with reduced structure observed in South and Central African regions. The cluster
451 3 of *C. sesamiae* located between Eastern and Western Rift Valley has an overlapping
452 distribution with "H" cluster of *B. fusca*. The cluster 2 on the East of Eastern Rift overlaps
453 with "KE" cluster of *B. fusca*. The cluster 4 of *C. sesamiae* ranges in East Africa at lower
454 altitudes where *B. fusca* is rare or absent (Dupas *et al*. 2014) and to the south. The clusters 4
455 and 5 exhibit large distributions that overlap with the "S" cluster of *B. fusca* from South to
456 East and Central Africa (Figure 2). A fifth population is also present in both species. Cluster
457 1 of *C. sesamiae* corresponds to parasitic wasp infecting *S. nonagrioides* that has been
458 described as a new species, *C. typhae*. Cluster "W" of *B. fusca* is only present in West Africa
459 and isolated from the other *B. fusca* populations (Figure 2). These results suggest that *B.*
460 *fusca* and *C. sesamiae* share a common phylogeographic history that explain the current
461 genetic structure of both species. For instance, the highest diversity for both species has been
462 found in the East African Rift Valley. The East African Rift valley also explained the
463 differentiation observed between two *C. sesamiae* lineages based on 6 mitochondrial and
464 nuclear markers (Kaiser *et al.*,2015). One lineage corresponds geographically and
465 ecologically to clusters 2 and 3, and the second one to cluster 4. The East African Rift Valley

has already been observed as a center of diversification for several species (Odee *et al.*, 2012; Habel et al, 2015; Freilich et al, 2016; Mairal *et al.*, 2017). This observed biological diversity has been related to both topological heterogeneity and variable climatic conditions that occurred since the formation of the East African Rift Valley ca. 20 Mya, with the alternation of arid and wet periods (Sepulchre *et al.*, 2006). Therefore, we could explain this observed pattern either by first the colonization of the East African Rift Valley followed by diversification or that the origin of both species lays in the East African East Valley which has been followed by further extension with admixture across Africa, except in West Africa, where *C. sesamiae* is absent and where *B. fusca* is totally isolated with zero migration observed to date (Sezonlin *et al.* 2006; Dupas *et al.* 2014).

Wolbachia *and biological control*

It is widely acknowledged that a better understanding of tritrophic interactions between plants, phytophagous insects and associated antagonists can help to develop better pest management strategies by identifying bottom-up and top-down effects in the food chain (Agrawal, 2000). *Wolbachia* can be considered as a fourth trophic level in such system but, the impact of *Wolbachia* on parasitoid host plant interactions has not received much attention. It was shown that a *Wolbachia* strain invasion temporarily reduces the impact of the parasitoid on its host (Branca & Dupas 2006). But this impact can be sustained in the case of stable contact between incompatible strains in “hybrid” zones. Conversely, *Wolbachia* can reinforce adaptive divergence between locally adapted populations to the benefit of the parasitoid (Branca *et al.* 2009). *Cotesia sesamiae* is a good model to test the effect of *Wolbachia* on host parasitoid assemblages as the four consensus genetic clusters differed for their *Wolbachia* and Lepidoptera host associations. In hybrid area, maladaptive gene flow may be observed and limited by *Wolbachia* strain bidirectional incompatibility. This is the

491 case between coastal (Mbsa) and inland (Kit) populations of the parasitoid (Dupas *et al.*
492 2008). The maladaptation may be the strongest in the AS Köppen Geiger Climate Zone
493 (corresponding to dry mid altitude agroclimatic zone) in wet seasons when *B. fusca*
494 represents half of the host community (Ong'amo *et al.* 2006), whereas avirulent *C. sesamiae*
495 toward *B. fusca* dominates parasitoid populations (Dupas *et al.* 2008). Strong counter
496 counter-selection of avirulent alleles is expected in *B. fusca* abundance peaks. *Busseola fusca*
497 is dominant in some seasons in mid altitude areas where virulent alleles dominate (Dupas *et*
498 *al.* 2008). Although avirulent parasitoids are able to select host at contact, which may reduce
499 maladaptation in the field, using contact cues to select host is risky because the host can bite
500 and kill the parasitoid before oviposition can be made; 25% of *C. sesamiae* entering the stem
501 tunnel are killed by *S. calamistis* larvae upon contact (Potting *et al.* 1999). The presence of
502 partially incompatible *Wolbachia* strains in the virulent and avirulent parasitoid populations
503 may favor their cohesiveness in balancing host communities across seasons. Hence, reducing
504 gene flow between locally adapted populations toward their host, in absence of premating
505 isolation, might reduce maladaptation in hybrid zone and our study confirms *Wolbachia* can
506 reinforce this process. For instance, very few heterozygous females between virulent and
507 avirulent alleles on the bracovirus CrV1 locus has been found in a previous study, since they
508 are likely maladapted (Branca *et al.*, 2011). Therefore, we would expect a lack of
509 recombination and strong diversification on genes, particularly at the bracovirus locus,
510 related to host specificity in *C. sesamiae*, pattern that has yet to be investigated at the genome
511 level.

512 Thompson (2005), in his seminal book on coevolutionary mosaics stressed that gene
513 flow had an ambivalent influence on coevolutionary interactions. Gene flow is essentially
514 maladaptive, bringing locally maladapted genes to populations in interaction (Nuismer 2006),
515 but in the presence of negative frequency dependent dynamics of coevolutionary interactions,

516 rare new variants originating from other populations may be adapted. Our results show some
517 congruence between *C. sesamiae* and *B. fusca* genetic structure (Dupas *et al.* 2014).
518 Congruence with host structure is therefore observed at different ecological levels, not only at
519 the level of host genus as shown by *adonis* analyses but also at the level of host populations.
520 This may reduce maladaptation of *C. sesamiae* toward *B. fusca* and favor local
521 coevolutionary interactions.
522
523 **Conclusion**
524 Our study presents a unique comprehensive case for assessing the determinant of
525 genetic structure in a parasitoid species, including multiple interactive biotic and abiotic
526 forces. The parasitoid, like its main host *B. fusca*, likely diversified across the East African
527 Rift Valleys where all the genetic clusters are found. Despite their wide distribution across
528 Sub-Saharan Africa, some populations have maintained their integrity as shown by the non-
529 spatial admixture model. Two important results pinpoint toward the strong influence of host
530 on parasitoids population dynamics and population genetics at a large geographical scale: (1)
531 although the species genetic clusters appear to have diversified across East African Rift
532 Valleys refuges, host species that are distributed across Africa became then the strongest
533 factor determining genetic structure, rather than climatic selection and geographic isolation
534 (2) migration rate inferred from Bayesian analysis of microsatellite data suggests a limitation
535 of gene flow due more to host adaptation than to *Wolbachia* infections. This result has a
536 fundamental importance in the context of biological control program. As opposed to chemical
537 control agents, biological control agents are expected to be able to cope with host evolution
538 (Holt & Hochberg 1997) but other interactions may limit this evolutionary sustainability. In
539 our case, parasitoid wasps are able to cope with host evolution despite many additional biotic
540 and abiotic ecological forces including reproduction manipulators that would be expected to

541 reduce local adaptation to host. The insect host dominates the piling up of all these factors
542 and could explain why parasitoids can be very successful biological control agents even when
543 introduced in climatically and geographically distant environments from their native settings
544 (Stiling & Cornelissen 2005). More generally, this work supports the hypothesis of the higher
545 impact of ecological *versus* neutral forces and of host *versus* other ecological forces on the
546 diversification of parasitoid - host interactions.
547

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Figure 1

Posterior probability of assignment of each individual of *Cotesia sesamiae* wasps to each of the 5 Instruct clusters and post-processed with CLUMPP (Cluster 1 in red, cluster 2 in yellow, cluster 3 in green, cluster 4 in blue, cluster 5 in purple). Individuals are grouped by the host genus where they were found. Individuals found on an unidentified host are not represented.

Figure 2

Distribution of genetic clusters of *Cotesia sesamiae* wasps for DAPC with K=5 (A), TESS3 software (B) and the Instruct software CLUMPP consensus with K=5 (C). For each clustering method, only individual with posterior probability of assignment above 0.5 are represented for each analysis. Distribution in Sub Saharan Africa is represented at the top and a zoom in Kenya at the bottom.

Figure 3

Distribution of *Wolbachia* infection in *Cotesia sesamiae* wasps across Sub-Saharan Africa. Red: wCsesA,: blue: wCsesA/wCsesB2, yellow: Absent, purple: wCsesB2, green: wCsesB1.

Figure 4

Estimates of gene flow between four geographic genetic clusters of *Cotesia sesamiae* wasps identified by Instruct. Each circle represents the infection status of individual found assigned to each cluster and the colour are corresponding to the one on figure 3. The fifth genetic cluster found only infecting *Sesamia nonagrioides* was excluded of the analysis.

Figure 5

Multiple correspondence analysis between microsatellite markers distance between individuals and ecological variables

Table 1. Status of the Kitale and Mombasa strains (Mochiah *et al*. 2002a)

Strain	Localisation	Major host association in the locality	Wolbachia status	Devt. Rate on *B. fusca*	Instruct Cluster
Kitale (Kit)	Inland Kenya	*Busseola fusca* (resistant)	wCsesB1	100%	5
Mombasa (Mbsa)	Coastal Kenya	*Chilo partellus* and *Sesamia calamistis* (susceptible)	WcsesA-wCsesB2	0%	2

Table 2 Analysis of molecular variance using microsatellite distance matrices and a full model containing all terms and interactions.

Factor	**Df**	**Sum of squares**	**F-Model**	**R^2**	**Pr(>F)**	
Host genus	8	43033	86.9617	0.37276	0.001	***
Wolbachia	4	13238	53.5037	0.11467	0.001	***
Köppen-Geiger climate	11	4896	7.1954	0.04241	0.001	***
Localization	13	9926	12.3438	0.08598	0.001	***
Host genus * *Wolbachia*	12	3735	5.0317	0.03235	0.001	***
Host genus *Köppen-Geiger climate	20	5042	4.0756	0.04368	0.001	***
Wolbachia * Climate	17	3261	3.1014	0.02825	0.001	***
Host genus * Localization	18	3071	2.7579	0.02660	0.001	***
Wolbachia * Localization	8	1603	3.2395	0.01389	0.001	***
Köppen-Geiger Climate * Localization	2	111	0.8950	0.00096	0.502	
Residuals	445	27526		0.24927		
Total	547	115442		1		

Table 3 Sum of squares and partial R^2 of Host genus, *Wolbachia* infection status, Köppen-Geiger climate and localization taken either as marginal effect or as the first term when adding them sequentially.

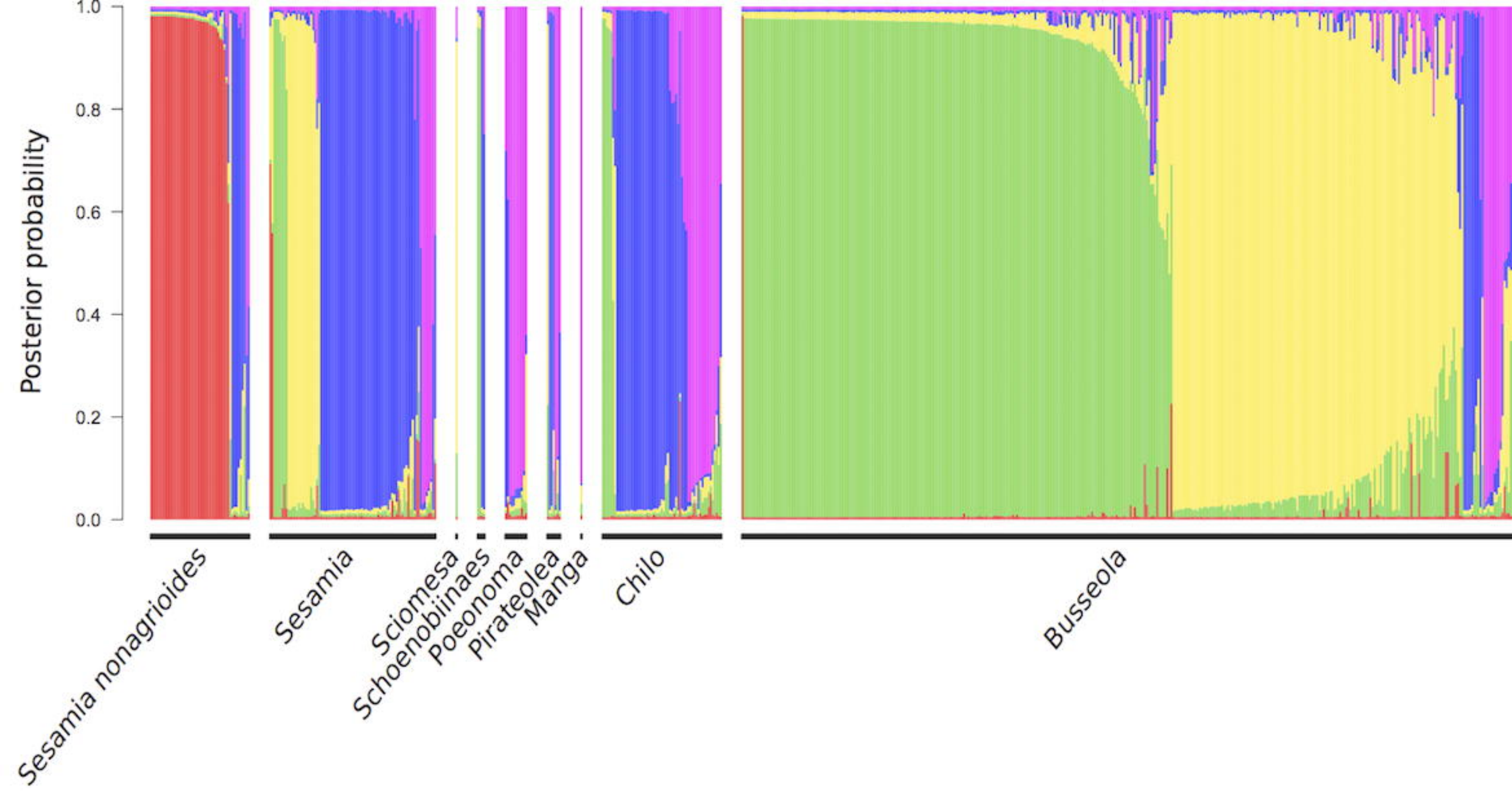
Factor	Df	Marginal Sum of squares	Marginal Partial R^2	1st Sequential Sum of squares	1st Sequential Partial R^2
Host genus	8	3709	0.03213	43033	0.37277
Wolbachia	4	5711	0.04947	49576	0.42944
Köppen-Geiger climate	11	2473	0.02142	32467	0.28124
Localization	13	9926	0.08598	34013	0.29463

Cross (Male x Female)	N	Brood size (mean ± SE)	N	Sex ratio (%Female, mean± SE)	N	Developmental time (days, mean ± SE)	N	Mortality outside the host		Mortality inside the host
								Number of dead cocoons (mean ± SE)	Number of dead larvae not forming cocoons (mean ± SE)	Number of dead larvae (mean ± SE)
Cs Kit cured x Cs Kit cured	28	34.0 ± 3.3b	28	48.8 ± 5.3a	28	18.5 ± 0.5a	28	2.3 ± 0.4a	2.2 ± 0.4b	0.8 ± 0.6a
Cs Kit cured x Cs Kit	25	36.0 ± 4.2ab	25	45.5 ± 4.5a	25	17.8 ± 0.2a	25	1.8 ± 0.4a	0.5 ± 0.2a	0.5 ± 0.2a
Cs Kit x Cs Kit cured	25	23.2 ± 3.0a	24	44.1 ± 5.7a	25	18.8 ± 0.4ab	25	3.8 ± 0.8ab	2.7 ± 0.6bc	1.0 ± 0.3ab
Cs Kit x Cs Kit	22	34.2 ± 3.1b	22	62.7 ± 5.4b	22	20.0 ± 0.4b	22	5.9 ± 0.9b	3.8 ± 0.5c	1.7 ± 0.4b
Cs Mbsa cured x Cs Mbsa cured	20	32.1 ± 3.9b	18	64.2 ± 7.2a	20	21.1 ± 0.4a	20	6.4 ± 1.1c	1.0 ± 0.3a	0.6 ± 0.2a
Cs Mbsa cured x Cs Mbsa	34	41.8 ± 4.3b	34	58.1 ± 4.4a	34	20.2 ± 0.3a	34	5.6 ± 1.1bc	2.6 ± 0.5a	1.0 ± 0.3a
Cs Mbsa x Cs Mbsa cured	19	21.4 ± 3.8a	16	55.1 ± 6.4a	19	21.0 ± 0.3a	19	3.5 ± 0.5a	1.5 ± 0.3a	1.3 ± 0.4a
Cs Mbsa x Cs Mbsa	24	38.9 ± 4.0b	23	57.2 ± 6.7a	24	21.4 ± 1.4a	24	5.4 ± 0.7b	3.5 ± 1.2a	1.0 ± 0.3a
Cs Kit cured x Cs Mbsa cured	25	27.3 ± 4.2a	20	68.1 ± 6.9ab	25	21.7 ± 0.6b	25	7.4 ± 1.0c	6.5 ± 1.4c	8.1 ± 1.4b
Cs Kit cured x Cs Mbsa	19	41.5 ± 3.9a	16	78.7 ± 5.5b	19	20.1 ± 0.2a	19	2.7 ± 0.7a	2.7 ± 0.5b	1.5 ± 0.4a
Cs Kit x Cs Mbsa cured	25	34.1 ± 5.3a	25	52.2 ± 4.8a	25	21.8 ± 0.4b	25	4.4 ± 0.5b	1.6 ± 0.4a	1.5 ± 0.4a
Cs Kit x Cs Mbsa	32	39.0 ± 3.4a	30	73.0 ± 3.5b	32	19.6 ± 0.3a	32	5.1 ± 0.9abc	4.1 ± 0.7bc	2.0 ± 0.6a

Cs Mbsa cured x Cs Kit cured	20	27.5 ± 4.0ab	17	47.8 ± 6.3c	20	19.0 ± 0.2b	20	2.1 ± 0.6a	3.0 ± 0.7ab	1.9 ± 0.6a
Cs Mbsa cured x Cs Kit	25	34.1 ± 4.9b	23	67.4 ± 4.5d	25	21.7 ± 0.4c	25	6.8 ± 0.6b	4.1 ± 0.6b	6.3 ± 1.1b
Cs Mbsa x Cs Kit cured	19	29.9 ± 5.6ab	17	11.6 ± 7.2b	19	18.1 ± 0.2a	19	3.3 ± 1.1a	2.8 ± 0.8ab	0.6 ± 0.2a
Cs Mbsa x Cs Kit	23	20.8 ± 3.3a	23	00.0 ± 0.0a	23	19.7 ± 0.3b	23	2.8 ± 0.7a	1.5 ± 0.3a	1.8 ± 0.6a

Table 4 Brood size, sex ratio, developmental time and mortality outside and inside the host of populations of different crosses on *Sesamia calamistis* (N = number of replicates).

Note. Cs Kit, *Cotesia sesamiae* from the inland Kitale area of Kenya; Cs Mbsa, *Cotesia sesamiae* from the coastal Mombasa area of Kenya; cured, *Wolbachia*-free parasitoids colonies (i.e. cured lines); in crosses within each population and between populations, values with different letter are significant (q-value <0.05; pairwise Wilcoxon's rank sum test, q-value = FDR corrected p-value).


Posterior probability
1.0
0.8
0.6
0.4
0.2
0.0
Sesamia nonagrioides
Sesamia
Sciomesa
Schoenobiinaes
Poeonoma
Pirateolea
Manga
Chilo
Busseola

TESS

Instruct

DAPC

Cluster 1 (*Sesamia nonagrioides*)
Cluster 2
Cluster 3
Cluster 4
Cluster 5

Google

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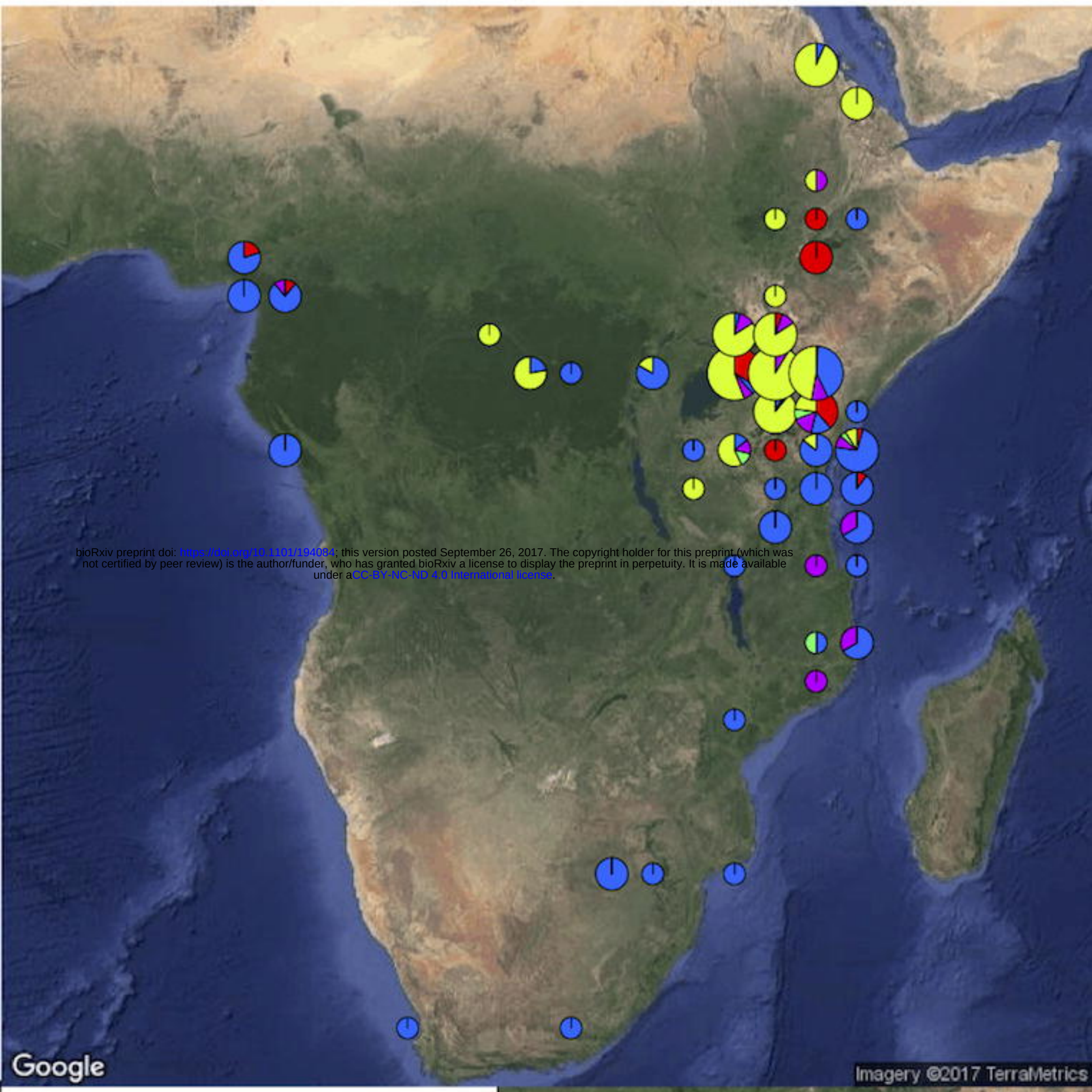

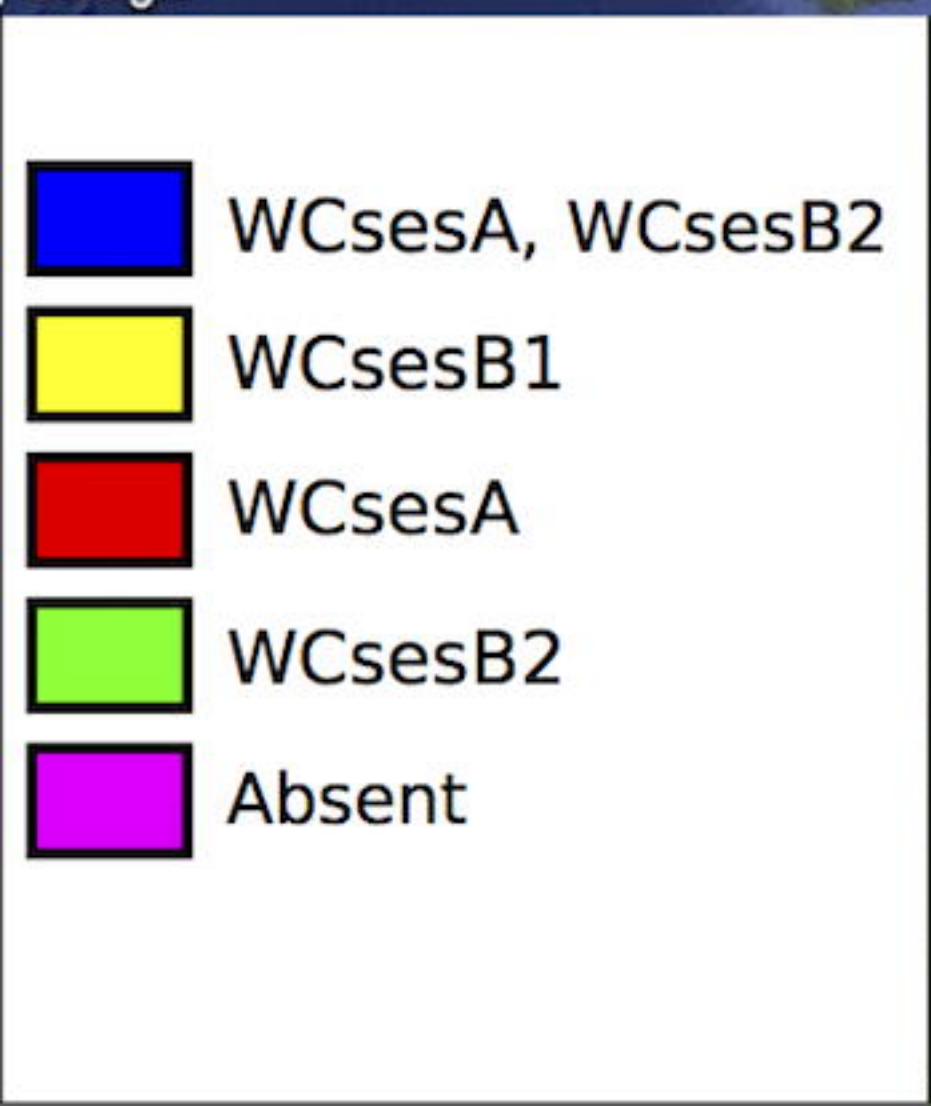

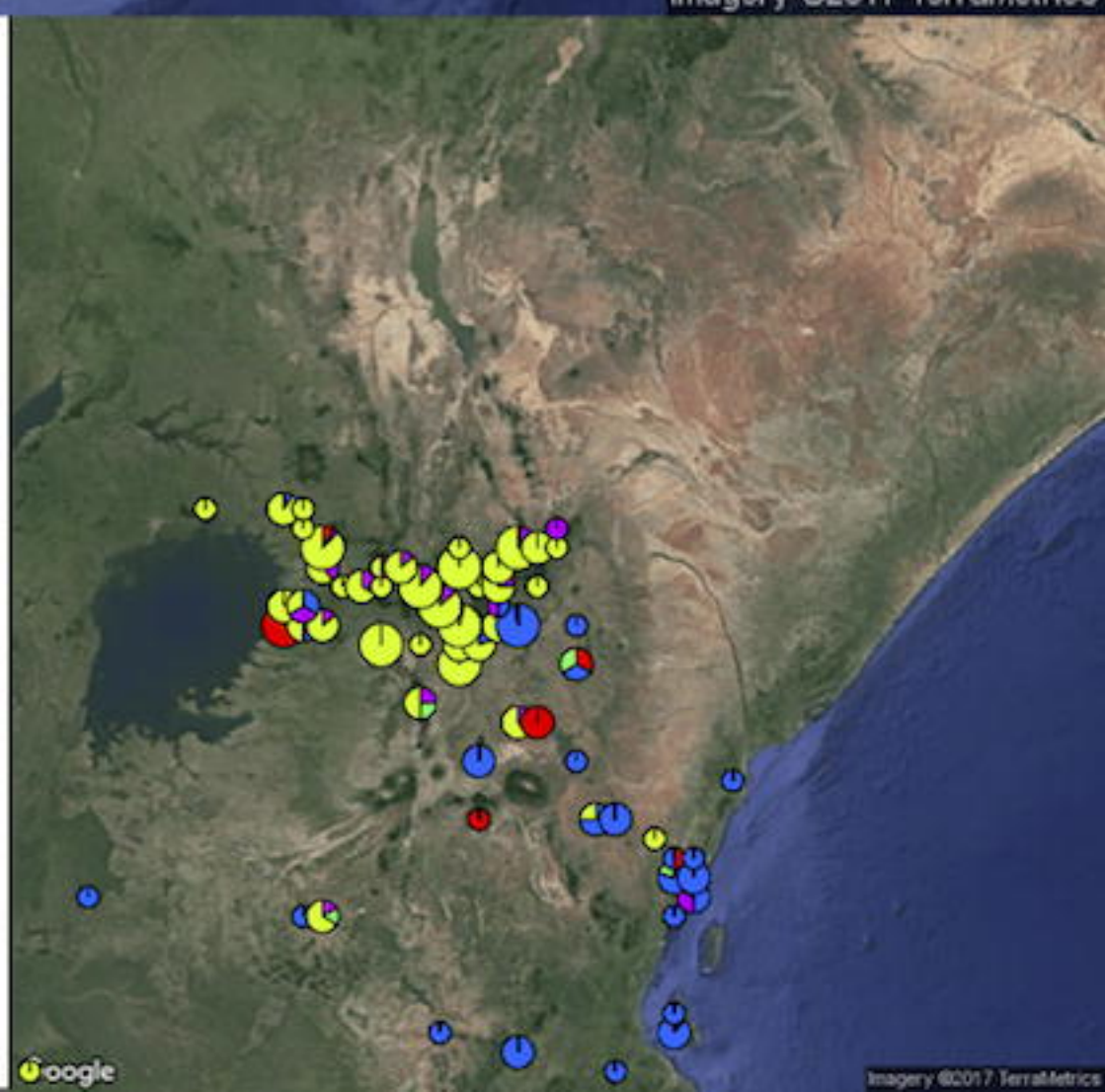

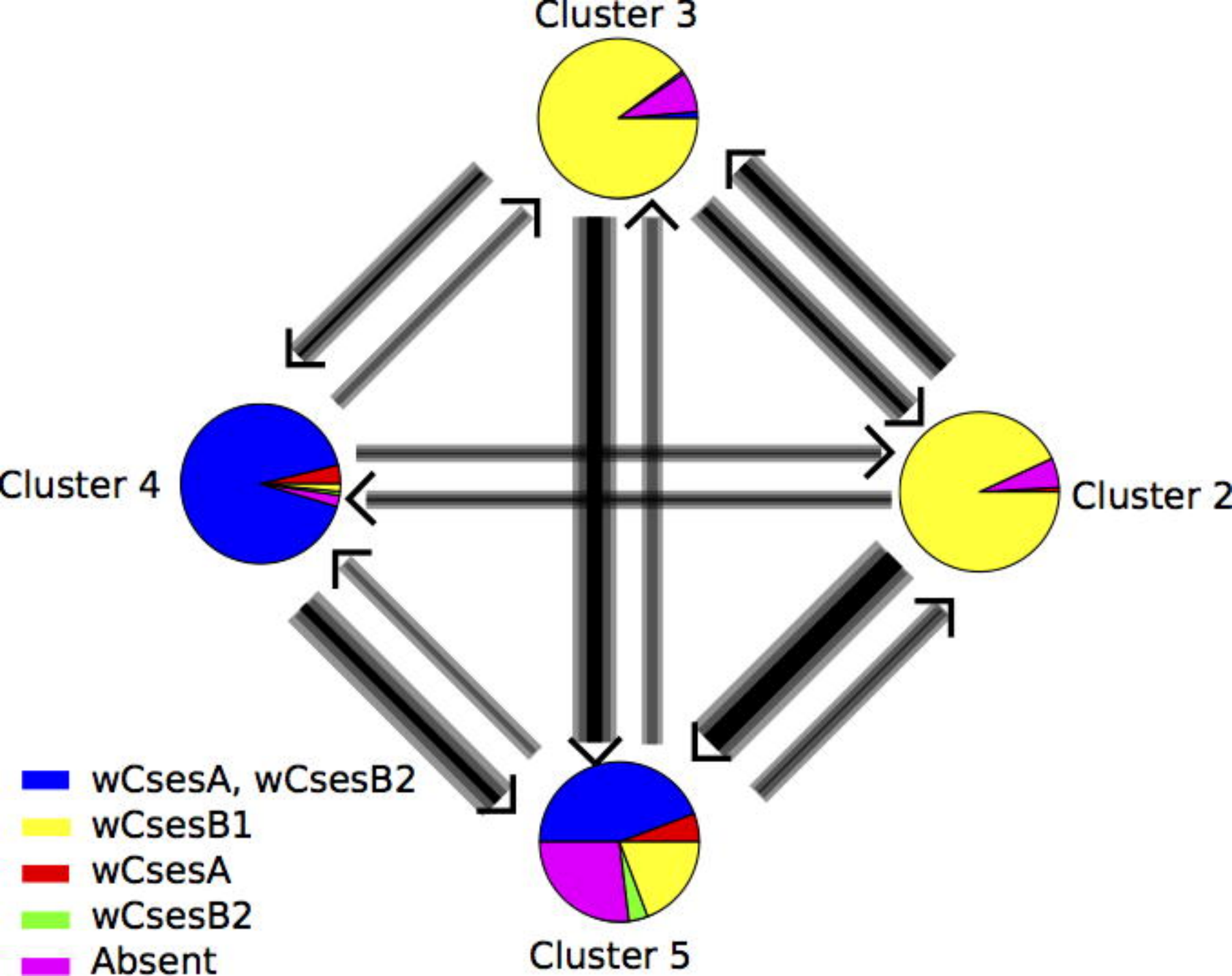
Cluster 3
Cluster 4
Cluster 2
Cluster 5
wCsesA, wCsesB2
wCsesB1
wCsesA
wCsesB2
Absent