Ann. soc. entomol. Fr. (n.s.), 2006, 42 (3-4) : 319-323 ARTICLE

Single-step PCR differentiation of *Cotesia sesamiae* (Cameron) and *Cotesia flavipes* Cameron (Hymenoptera: Braconidae) using polydnavirus markers

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Abstract. *Cotesia sesamiae* (Cameron) and *Cotesia flavipes* Cameron (Hymenoptera: Braconidae) are the main larval parasitoids of cereal stemborers in sub-Saharan Africa. *Cotesia sesamiae* is endemic to eastern and southern Africa, while *C. flavipes* was introduced into the region for biological control against the exotic lepidopteran *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae). The two are sibling parasitoids, difficult to distinguish morphologically. The introduced insect could potentially lead its African biotype to extinction because of their similar ecological niche. In order to distinguish the two species, multiplex primer-specific and PCR-RFLP tests were developed. Rapid identification of the two species was possible using primer-specific tests on DNA extracts as well as on pieces of tissue in a single PCR step followed by gel electrophoresis. The CRV1 gene of the polydnavirus, a symbiont to the wasps, was used as the marker. The results show that the morphological identifications, validated by molecular tests, are accurate in 93% of cases.

Résumé. Simple analyse par PCR pour distinguer *Cotesia sesamiae* (Cameron) (Hymenoptera : Braconidae) de *Cotesia flavipes* Cameron (Hymenoptera : Braconidae) en utilisant des marqueurs du polydnavirus. *Cotesia sesamiae* (Cameron) et *C. flavipes* Cameron (Hymenoptera : Braconidae) sont parmi les principaux parasitoïdes larvaires des foreurs de tige de céréales cultivées en Afrique Sub-Saharienne. L'un est endémique, l'autre fut introduit à des fins de lutte biologique contre un lépidoptère exotique foreur de tige, *Chilo partellus* (Swinhoe). Les deux parasitoïdes sont difficilement distinguables morphologiquement. *Cotesia flavipes* présente une niche écologique similaire à l'un des biotypes de *C. sesamiae*. Il peut potentiellement le menacer d'extinction. Afin de distinguer les espèces à l'aide de tests fiables et de mieux suivre l'évolution de leurs effectifs, nous avons développé un test PCR-RFLP et un test multiplex-amorce spécifique. Le test amorce-spécifique a permis l'identification rapide des deux espèces à partir d'ADN extrait ou de morceaux de tissus en une seule étape de PCR suivie d'une électrophorèse sur gel. Les résultats montrent que les identifications morphologiques ne sont correctes que dans 93% des cas.

Keywords: Species differentiation, *Cotesia flavipes*, *Cotesia sesamiae*, multiplex PCR, polydnavirus.

Classical biological control aims to stabilize ecosystems that have been destabilized by the introduction of exotic invaders or by intensive agriculture. It is based on the beliefs that the invaders' ecological success (usually, the pests' ecological success) is due, at least partly, to the absence of natural enemies in the environment, and that the introduction of natural enemies will allow the system to return to equilibrium. However the biological control agent is itself an invader in the system, and can be unsafe for the ecosystem also (Holt & Hochberg 2001; Hopper 2001; Waage 2001). In many cases, antagonist species closely related to the introduced agent are present and are difficult to distinguish morphologically (Perdikis *et al.* 2003). In order to follow up introductions and better evaluate the effect of biological control on the natural endemic fauna of antagonists, it is necessary to develop rapid taxonomic diagnostic tools (Hinz *et al.* 2001).

Cotesia sesamiae (Cameron 1891) and *Cotesia flavipes* Cameron 1891 (Hymenoptera: Braconidae) are the main larval parasitoids of cereal stemborers in eastern and southern Africa. *Cotesia sesamiae* is endemic to Africa and *C. flavipes,* a native to Asia, has been introduced repeatedly in Africa since 1993 as a biological control agent to control the exotic stemborer *Chilo partellus* (Swinhoe 1885) (Lepidoptera: Crambidae) (abbreviated as *Ch. partellus* in this paper). The pest originates from India but was introduced into Africa in the 1930s. *Cotesia flavipes* is now widespread over eastern and southern Africa. Both parasitoids have similar ecological niches. *Cotesia sesamiae* exists

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Accepté 29 juin 2006

in two biotypes, one of which is virulent to *Busseola fusca* (Fuller 1901) (Lepidoptera: Noctuidae), while the other, like *C. flavipes,* is avirulent and unable to develop on *B. fusca* (Ngi-Song *et al.* 1998). On *Ch. partellus*, the intrinsic rate of population increase of *C. flavipes* was higher than *C. sesamiae* at all temperatures, suggesting that the exotic parasitoid is able to respond faster to host density changes (Mbapila 1994). Due to the higher rate of parasitism of *C. flavipes* on *Ch. partellus*, there is the possibility that *C. flavipes* might drive the avirulent biotype of *C. sesamiae* to extinction in areas where *B. fusca* is absent. On the other hand, it has been observed that *C. flavipes* is attracted more strongly to infested maize, while *C. sesamiae* exhibits a preference for infested sorghum (Ngi-Song *et al.* 1996); these differences may prevent competitive displacement. This entomological risk is a question that needs to be well documented and understood for the sustainability of biological control programmes (Hopper 2001; Waage 2001). The exotic *C. flavipes* is now the dominant parasitoid in the coastal belt of Kenya where *Ch. partellus* is dominant (Zhou *et al.* 2003), but no evidence from the field was found of the competitive exclusion of *C. sesamiae* by *C. flavipes* (Sallam *et al.* 2001). Since it is expected that the rate of parasitism will depend on the prevalence on each plant and stemborer species, field data are needed on the abundance of *C. flavipes* and *C. sesamiae* under these different ecological conditions.

One difficulty in these surveys is that the two species are very difficult to distinguish morphologically (Kimani-Njogu & Overholt 1997). A fast diagnostic test is needed.

Previous studies have shown that infrared spectroscopy can distinguish the cocoons of the two species with an accuracy of more than 85% (Cole *et al.* 2003). Molecular markers may also be of use. Polymerase chain reaction (PCR) amplification is a powerful technique for species diagnosis that has been used for identifying pathogens and parasites, and for distinguishing between sibling species of macroscopic, free-living organisms. The best known technique, PCR-RFLP, is based on specific digestion of a diagnostic amplimer. It is highly reliable and therefore widely used, especially with the development of barcoding systematics (Blaxter *et al.* 2005). Another technique, based on allele-specific PCR and primer-induced fragment-length variation, appears more cost effective and powerful. It uses one forward primer that is common to all targets and one reverse specific primer that anneals at different positions for each target. The amplimers are separated directly by gel electrophoresis. Direct amplification from pieces of tissue is also used to accelerate the technique and reduce its cost.

Polydnaviruses are obligatory symbionts of many braconid and ichneumonid parasitoid wasps. In braconids, symbiosis is dated at ca. 73 million years ago and involves around 17 500 species of the microgastroid complex. The virus is transmitted from one generation to the next on wasp chromosomes; no horizontal transmission has been detected so far. The perfect parallel phylogeny observed between wasp and polydnavirus genes is consistent with the premise that these symbionts can be considered as other nuclear genes (Whitfield 2000). Nevertheless, molecular investigations suggest that the rate of substitution of polydnavirus genes is accelerated by positive Darwinian selection and by increased mutation rates compared to other nuclear genes (Dupas *et al.* 2003). This makes them suitable markers at low phylogenetic levels, within species, or especially for distinguishing between closely related species. In addition, the polydnavirus markers are pre-amplified in the wasps' ovaries through a virus replication process, which makes them easier to detect by PCR.

Cotesia rubecula (Marshall 1885) (Hymenoptera: Braconidae) CrV1 is a polydnavirus gene involved in host immune suppression by targeting host haemocytes. The aim of this work was to develop a PCR test for the identification of the two species of stemborer parasitoids based on sequence differences in the polydnavirus gene CrV1 between *C. sesamiae* and *C. flavipes*. Two techniques were used, one based on PCR-RFLP and one based on primer-specific direct PCR fragment-length differences.

Material and Methods

Insect sampling

Specimens originating from 97 localities in sub-Saharan Africa (Kenya, Tanzania, Congo) and three localities in Asia (India and Pakistan) were used in the analyses. Some individuals were sequenced for the CrV1 marker in order to develop a molecular test; others were used directly for the PCR tests. The eight *C. flavipes* individuals sequenced came from India (1 locality), north and south Pakistan (1 locality each) and Kenya (5 localities); the 18 *C. sesamiae* used for sequencing were from Congo (3 localities), Kenya (12 localities) and Tanzania (3 localities). Additional individuals were used for the PCR tests. A total of 28 *C. flavipes* from 17 localities in Kenya, and 205 *C. sesamiae* from 84 localities across sub-Saharan Africa (16 localities in Congo; 65 localities in Kenya; 3 localities in Tanzania) were genotyped either by sequencing, by PCR tests or both.

Morphological identification

Morphological identification was performed on one or two males per cocoon mass, based on the morphology of the genitalia (Kimani-Njogu & Overholt 1997).

Molecular characterization

Whole insect bodies were frozen in liquid nitrogen for 1 min and ground in a mortar and pestle. Different extraction protocols were used for the sequencing and PCR tests. For sequencing, total DNA was extracted using a DNEasy tissue Kit (Qiagen Gmbh). For the PCR tests, individuals were extracted using the Chelex® (BioRad) protocol, or the PCR was performed directly on non-crushed bodies or on the last three to four abdominal segments. For Chelex extraction, 100 µl 5% Chelex was added to the ground insect and mixed with a mortar and pestle. Protein-Chelex adhesion was performed during two cycles of 10 min each at 99 °C and separation was done by three tube inversions at room temperature.

For sequencing, a 999 bp fragment of the CrV1 polydnavirus gene was amplified using primers CrV1087F: 5'ATGTCACTCGTCAAAAGTGC3' and CrV2107R: 5'AAAGTTTGCGATGGGGTTGT3' designed from the *C. rubecula* CrV1 gene sequence and named according to their positions in the GeneBank sequence AF359344. Another set of primers CsfV1125F: 5' TCTCCTGTGTCAATCATGTAAGTT3', and CsfV1955R: 5'ACTCCTTCAACGCTGGGTTCCTTG3', were designed from the *C. sesamiae* and *C. flavipes* sequences obtained in the present study. The PCR cycling conditions were as follows: initial denaturation 5 min at 94 °C, 40 cycles of 50 s at 94 °C, 1 min 20 s at 51 °C (52 °C for CsfV1125F-CsfV1955R primer pair) and 1 min 20 s at 72 °C. Final extention was 10 min at 72 °C. The reaction mixture contained 0.4 µM primers, 0.24 µM dNTP, and 1µl DNA plus 1 Taq T4 DNA polymerase (Promega) per 25 µl of reaction. The $MgCl_2$ concentration was 2 mM for the CrV1087-CrV2109 primer pair and 1.5 mM $MgCl_2$ for the CsfV1125F-CsfV1955R primer pair. Sequencing reactions were performed in both directions using the amplification primers on an automated sequencer (ABI PRISM 3100).

A primer-specific direct PCR test was developed based on the CrV1 sequences, with two forward primers designed to anneal specifically to *C. sesamiae* and *C. flavipes* sequences at different positions and one reverse annealing to both (fig. 1). The *C. sesamiae*-specific primer was CsV1394F 5'AACGAACACTTTCGATGAA3' and the *C. flavipes*-specific primer was CfV1634F 5'GAGTATTTTCCGAAAATGG3'. The reverse, non-specific primer was Csf1955R. Expected amplimer sizes based on *C. rubecula* sequences were 511 bp and 271 bp for *C. sesamiae* and *C. flavipes*, respectively. PCR cycling was the same as above except for the annealing temperature, at 60 °C. The reaction mixture contained 0.28 µM primers, 0.24 µM dNTP, and 0.5 µl DNA plus 0.5u Promega® Taq DNA polymerase per 12.5 µl of reaction. Reactions were performed either on Chelex extracts or directly on non-extracted tissues. For each PCR reaction, a negative control (water) and positive controls (confirmed *C. flavipes* and *C. sesamiae* DNA extracts) were performed. Amplimers were loaded on a 1% agarose-0.5% TBE gel next to Invitrogen® 100bp ladder. Migration was performed in 0.5% TBE during 30 min at 100V on an *i*-mupid® (Eurogentec) gel migration system.

Repeatability and discrepancies between morphological and molecular identifications

When different individuals from the same cocoon mass are used for the morphological and molecular identification, any discrepancies may be due to multiparisitism by *C. flavipes* and *C. sesamiae* (i.e. two species in the same cocoon mass) (Ngi-Song *et al.* 2001). Therefore, in the present study when molecular and morphological identifications were different, other individuals of the cocoons were tested by PCR to confirm that the cocoon mass did not contain a species mix of *C. flavipes* and

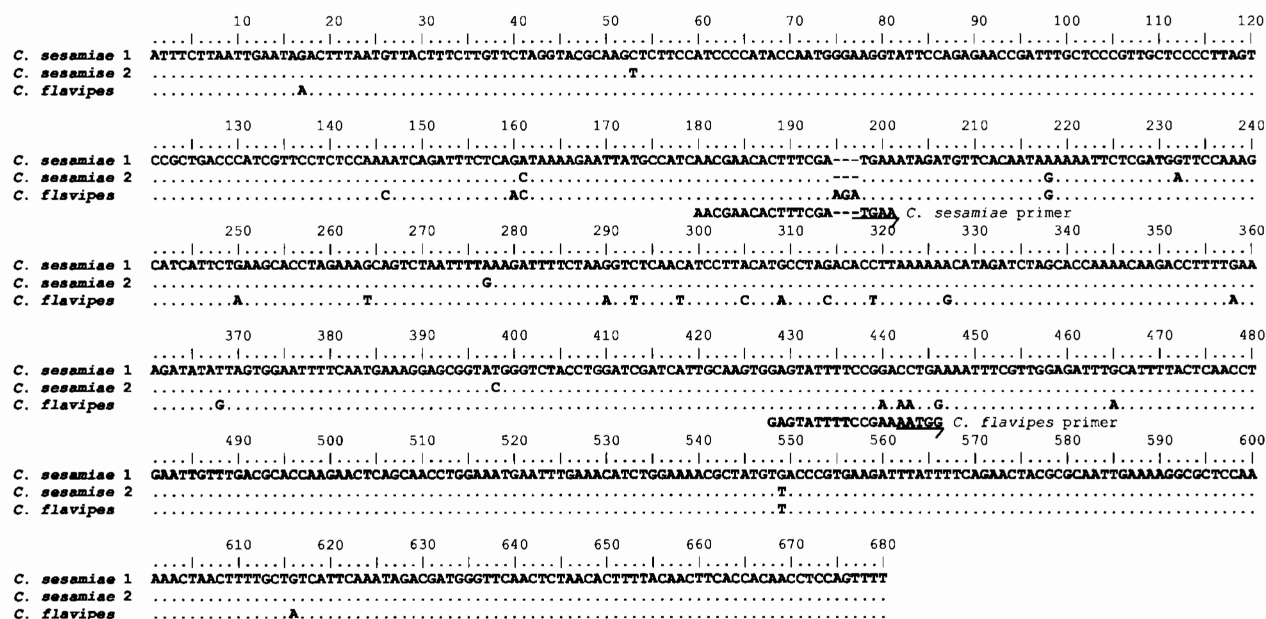

Figure 1
Cotesia sesamiae and *Cotesia flavipes* polydnavirus CrV1 haplotypes, and species-specific primers.

C. sesamiae. In addition, the Cytochrome *b* mitochondrial gene was sequenced on six of the individuals showing discrepancies (P89 Kisumu 1, P172 Rift Valley 3, P77 Mt Kenya 1, P210 Mt Kenya 2, P80 Mt Kenya 3, and P 162 Mombasa 6, all from Kenya). The primers were CB1 and CB2 (Simon *et al.* 1994). PCR annealing was done at 48°C with 3 mM $MgCl_2$. All other conditions were as for CrV1.

A PCR test was also developed based on CrV1 sequences. Enzyme Fok I was found to cut the CsfV1125F-CsfV1955R amplimer once at position 76 for *C. flavipes* and twice, at positions 76 and 374, for *C. sesamiae*. Expected fragment sizes based on the sequences were 76 bp, 298 bp and 456 bp for *C. sesamiae* and 76 bp and 754 bp for *C. flavipes*. This test was developed as an alternative to the primer-specific test, but was not used in this study.

Results

A fragment of 680 bp encoding CrV1 was sequenced from eight *C. flavipes* and 19 *C. sesamiae* individuals from one locality each, over their entire natural distribution range. We observed three haplotypes, one in *C. flavipes* and two in *C. sesamiae* (GeneBank accession numbers DQ356254 to DQ356256) (fig. 1). The divergence was 1.0% between *C. sesamiae* haplotypes and 3.5% and 3.7% between the *C. flavipes* haplotype and the two *C. sesamiae* haplotypes. Based on this sequence survey, diagnostic sequence variations were selected to distinguish the two species (fig. 1). A primer-specific test was developed. PCR amplification led to one band around 600 bp for *C. flavipes*, or one band around 300 bp for *C. sesamiae*, as expected from the sequencing data.

We investigated 233 cocoon masses for discrepancy between mophological and molecular identifications, of which 28 had been attributed to *C. flavipes* (noted *Cf* in this paragraph) morphologically and 205 to *C. sesamiae* (*Cs*). The type PCR gel obtained is presented in fig. 2. Among the 28 individuals identified as *Cf* morphologically, 25 were *Cf* and 3 were *Cs* based on the PCR tests. Among the 205 morphological-*Cs*, 192 were *Cs* and 13 were actually *Cf* based on the PCR test (tab. 1). Some of the cocoon masses identified morphologically as *Cs* but characterized as *Cf* with the PCR test were analysed in more detail. Other cocoons were taken from the cocoon mass and run for PCR tests. Two of the eight cocoon masses were shown to be a *Cf* + *Cs* species mix, and the remaining six proved to be pure *Cf* (tests were performed on 3 to 7 additional individuals from the cocoon masses showing discrepancies) by PCR. The reliability of the PCR test on CrV1 was confirmed by sequencing the Cytochrome *b* fragment of six individuals (see Material and Methods) that had been identified as *Cs* morphologically but as *Cf* on PCR tests. All were confirmed as *Cf* based on the Cytochrome b sequence (GeneBank DQ459001, Dupas unpublished data).

Some PCR tests were also performed on pieces of tissue without DNA extraction. The results, presented in fig. 2, show that the reaction can be performed effectively on abdomen ends, cut from either alcohol-preserved or dried specimens, but not as satisfactorily on non-crushed whole bodies. Since we were confident of the molecular tests, the rate of morphological misidentification was calculated based on the results of the molecular tests. Among the entire sample tested, *Cf* was more likely to be considered morphologically as *Cs* than *vice versa;* 13 of the 37 individuals genotyped as *Cf* had been identified morphologically as *Cs* (35.1 % error), while only three of the 195 genotyped *Cs* individuals had been identified as *Cf* morphologically

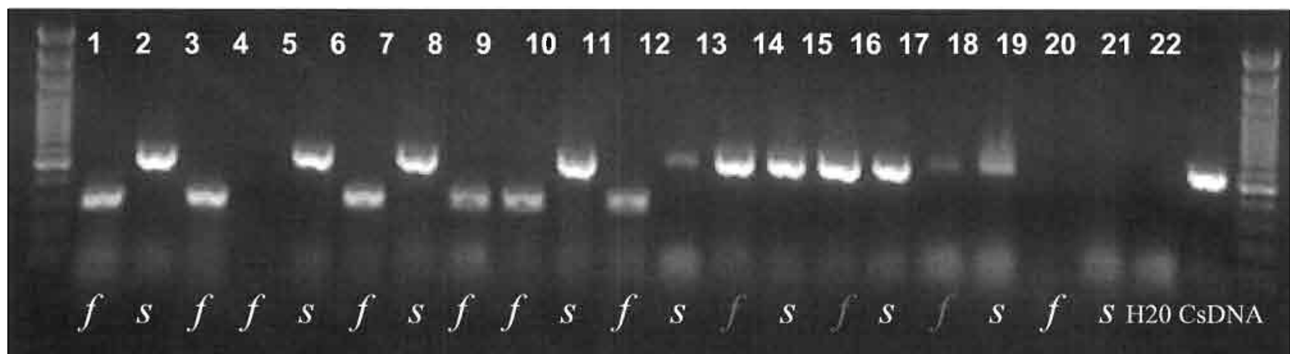

Figure 2
Results of PCR test performed on non-extracted DNA material (lanes 1-20): cut abdomen ends (lanes 1-16) or whole body (lanes 17-20). Individual specimens were either preserved in fresh alcohol (lanes 1-7), dried within the previous month (lanes 8-12), alcohol-preserved for 3 years (lanes 13-14), dried for 3 years (lanes 15-18), or freshly dried (lanes 19-20). Lane 21, negative control. Lane 22, *Cs* DNA extract positive control. "*s*" was identified as *Cotesia sesamiae* and "*f*" identified as *Cotesia flavipes* morphologically. The *C. sesamiae* band co-migrates with the 500 bp length marker, and the *C. flavipes* band with the 300 bp length marker. Samples for which molecular identification differs from morphological identification are indicated in grey.

(1.5% error). Overall, the reliability of morphological identification was 93.1%.

Discussion

A PCR test based on species-specific CrV1 polydnavirus gene primers was designed to distinguish between the sibling species *C. flavipes* and *C. sesamiae*. The results showed the reliability and rapidity of this method. The test consists of a single PCR followed by gel electrophoresis visualisation using a mix of three primers and performed on extracted DNA or even non-extracted pieces of tissues from dried or alcohol-preserved specimens of either sex. The viral nature of the genes and their localization in the calyx of the female increases the number of copies and may improve the successful application of PCR amplification, allowing for a fast, single-tube extraction PCR procedure, even from non-extracted abdomen ends.

The PCR tests developed here are based on a survey of *C. sesamiae* and *C. flavipes* sequences present in central and eastern sub-Saharan Africa. The survey did not include West African *C. sesamiae*. Although *C. sesamiae* is rarely found in that region, it remains to be confirmed whether the PCR test can be used for insects collected there. The *C. flavipes* populations included in the analyses were either from sub-Saharan Africa or from the insects collected in India and Pakistan for release in eastern and southern Africa. The reliability of the PCR tests in differentiating the two species remains to be checked for other regions.

Molecular tests showed that the insects had been correctly identified from male genitalia criteria with a 93.1% reliability. Morphological identification may be good enough in many situations, but molecular testing may be useful to estimate the morphological identification bias toward one or the other species in post-release surveys.

Acknowledgements. This study was supported by IRD UR072. We thank members of the ICIPE Noctuid Stemborer Biodiversity Project, especially Boaz Muzyoka, Leonard Ngala and George Ong'amo for technical assistance during insect collection and insect rearing in the ICIPE laboratories in Kenya. We also thank Hellen Mwadime of the Cereal Stemborers Project for the morphological identification of the field-collected insects.

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