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GENETIC DISCRIMINATION OF TWO COWPEA (*VIGNA UNGUICULATA* (L.) WALP) BRUCHID (COLEOPTERA, CHRYSOMELIDAE, BRUCHINAE): *CALLOSOBRUCHUS MACULATUS* (F.) AND *BRUCHIDIUS ATROLINEATUS* (PIC.)

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ABSTRACT: The cowpea, *Vigna unguiculata* (L.) Walp is a food leguminous plant cultivated in tropical Africa for its seeds rich in proteins. The main problem for its production is the conservation of the harvests. In the fields as in the stocks, the seeds are destroyed by pests (bruchids). *Bruchidius atrolineatus* Pic. and *Callosobruchus maculatus* Fab. (Coleoptera-Bruchidae) are the most important pests of cowpea beans. Cowpea infestation by these two bruchid species starts in the field when the plant begins to bear fruit and continues through storage, resulting in substantial damage if no control action is taken, they can destroy 80 to 100% crop after several months of storage. The objective of this work is to develop a fast and reliable identification method of these species of Bruchidae through PCR-RFLP. Primary outcomes were recorded allow to consider that portion of DNA amplified with our primer pairs has no restriction site for the enzyme *Bsm*AI in *Bruchudius atrolineatus* both cytochrome b and for the 28S. For *Callosobruchus maculatus* with the same restriction enzyme, DNA is cut into 3 fragments for the cytochrome b gene and 2 for the 28S.

Keywords : Cowpea, *Callosobruchus maculatus*, *Bruchidius atrolineatus*, PCR-RFLP, C*ytb* DNA, 28S, *Bsm*AI

INTRODUCTION

Cowpea,*Vigna unguiculata* (L.) Walpers are cultivated in West and Central Africa mostly for the edible seeds which serve as an important source of protein in the human diet. In Senegal, it is the second leguminous plant after groundnut. As a basic and appreciated food, it is also the cheapest food which provides proteins to most of the rural people [7] .However, as in the case of many other food crops, a wide spectrum of insect pests attack cowpea both in the field and in storage causing severe economic damage [10; 1; 2]. Among them, the beetles, *Callosobruchus maculatus* (F.) and *Bruchudius atrolineatus* cause most of damage. Adult beetles lay eggs on pods in the field or seeds in storage. Larvae bore into and feed within seeds causing weight loss, decreased germination potential and a reduction in commercial [1; 2] .Beetle populations build up rapidly in storage and damage in terms of holed seeds can increase to 99% after 6 months in Senegal [11]. *B. atrolineatus* is present in the crops, but is not adapted to storage systems. From the first generations of storage, over 90% of adults are in reproductive diapause and leave attics often badly closed. As against adults of *C. maculatus* is maintained throughout the dry season and produces up to sixteen generations in a year when the seeds are available [5] and cause losses estimated at between 800 to 900 g / kg in different areas in West Africa [9; 3].

These two species of Bruchidae are sympatric and some authors have reported biological abilities that are observed when conditions become unfavorable with reproductive quiescence in *C. maculatus* [8] and reproductive diapause characterized both in *B. atrolineatus* [4] and *C. maculatus* [13].
The objective of this work is the molecular identification of these insects Bruchidae subservient to cowpea (*Vigna unguiculata* L. Walp) by PCR-RFLP.
Molecular genetic methods have several advantages over classical morphological and chemical analyses. For instance, the genetic method requires genotype instead than phenotype, therefore DNA based experiments have become widely employed techniques for a rapid identification. For these reason, we developed molecular tests allowing for an easy and rapid discrimination among the 2 species. The tests were based on the use of restriction sites generating specific profiles of cytochrome b and 28S DNA sequences

MATERIEL AND METHODS

Samples

All specimens were collected in several fields in Senegal. Bruchids used for molecular analysis were preserved in pure ethanol.
DNA extraction and PCR amplification
Total genomic DNA was extracted from prothorax of individual seed-beetles as a Qiagen, DNeasy Blood & Tissue Kit. A partial Cytochrome b (*Cyt b*) end region was amplified using the primers CB1 (5'–TATGTACTACCATGAGGACAAATATC 3') and CB2 (5' ATTACACCTCCTAATTTATTAGGAAT-3').
The 28S ribosomal DNA was targeted for PCR, amplified and sequencing with primer D2CF D45F (5'-TAC CGT GAG GGA AAG TTG AAA-3 ') and D2CR D45R (5'-AGA CTC CTT GGT CCG TGT TT-3'). For both markers, PCR amplification were performed in 25 µl reaction volume 2.5 µl enzyme buffer supplied by the manufacturer, 2.5 mM MgCl2, 0.6 unit of Taq polymerase (Promega), 17.5 pM of each primer, 25 nM of each DNTP and 1µl of DNA extract. After an initial denaturation step at 92°C for 3 min, reaction were subjected to 35 cycles for 1 min at 92°C, 1 min 30 s at 48°C and 1 min at 72°C. PCR products (5 µl) were checked on a 1,5% agarose gel in the presence of ethidium bromide and visualized under UV light.

Design of the restriction method
Sequences of the Cyt B and 28S gene were downloaded from Genbank and aligned using ClustalW [12] as implemented in BioEdit and attachment sites of our primers were searched. The species and sequences were as follows:

- *Decellebruchus (Bruchidius) atrolineatus Cytb* : accession AY625470; version AY625470.1 GI:55276683 ;
- *Decellebruchus (Bruchidius) atrolineatus* 28S : accession AY625371; version AY625371.1 GI:55276493 ;
- *Callosobruchus maculatus Cytb* : accession AY625466; version AY625466.1 GI:55276675 ;
- *Callosobruchus maculatus* 28S: accession AY625367; version AY625367.1 GI:55276489.

Virtual restriction digest were performed using NEBcutter (http://tools.neb.com/NEBcutter2/index.php) to select useful restriction enzymes.

Digestion by restriction enzymes and recording of the fragment length polymorphisms

Aliquots of about 10 µl of PCR products were digested separately for 2 h at 37°C with the restriction enzyme *BsmAI* and 100 µg /ml BSA (BioLabs) according to the manufacturer instructions.

The digested DNA fragments were separated on 2.5% agarose gel in the presence of ethidium bromide and visualized under UV light; 200 bp DNA ladder (BioLabs) was used to estimate the DNA fragments sizes.

RESULTS AND DISCUSSION

The successfully amplification, produce approximately a 600-bp fragment, for the *cyt b* and 28S DNA (Fig 1A; 1B).

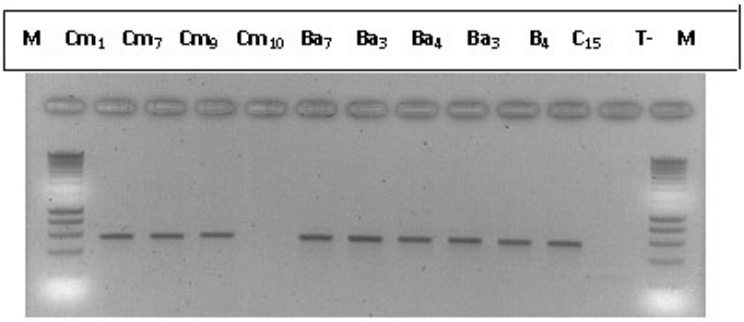

Figure 1A: 28S PCR products before digestion. Cm= *C.maculatus*, Ba= *B. atrolineatus,* the number next represents the individual

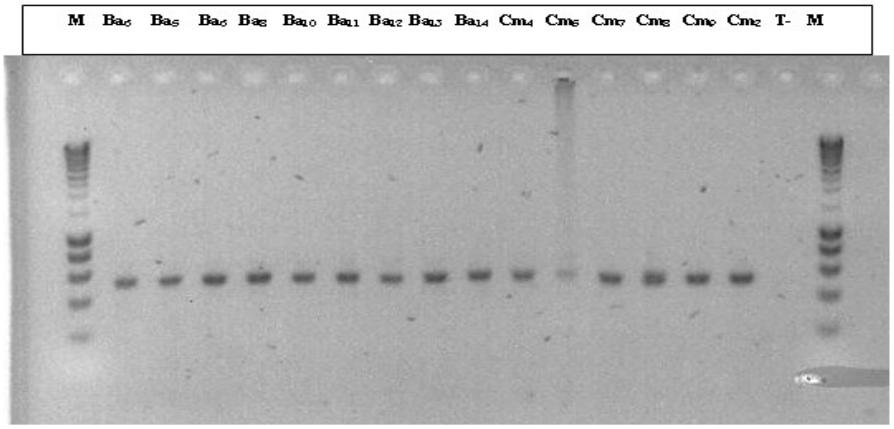

Figure 1B : Cytochrome b PCR products before digestion. Cm= *C.maculatus*, Ba= *B. atrolineatus,* the number next represents the individual

The electrophoretic patterns of PCR products digested with restriction enzyme *Bsm*AI are shown in Figure 2. Judging from the *28S DNA* digestion fragments, the bruchidea species could be separated to 2 groups, digested and nondigested. At same, based on *cyt b* gene, we had 3 restrictions profiles. The products of restriction are shown separately in figure 2 for 28S and figure 3 for *Cytb*.

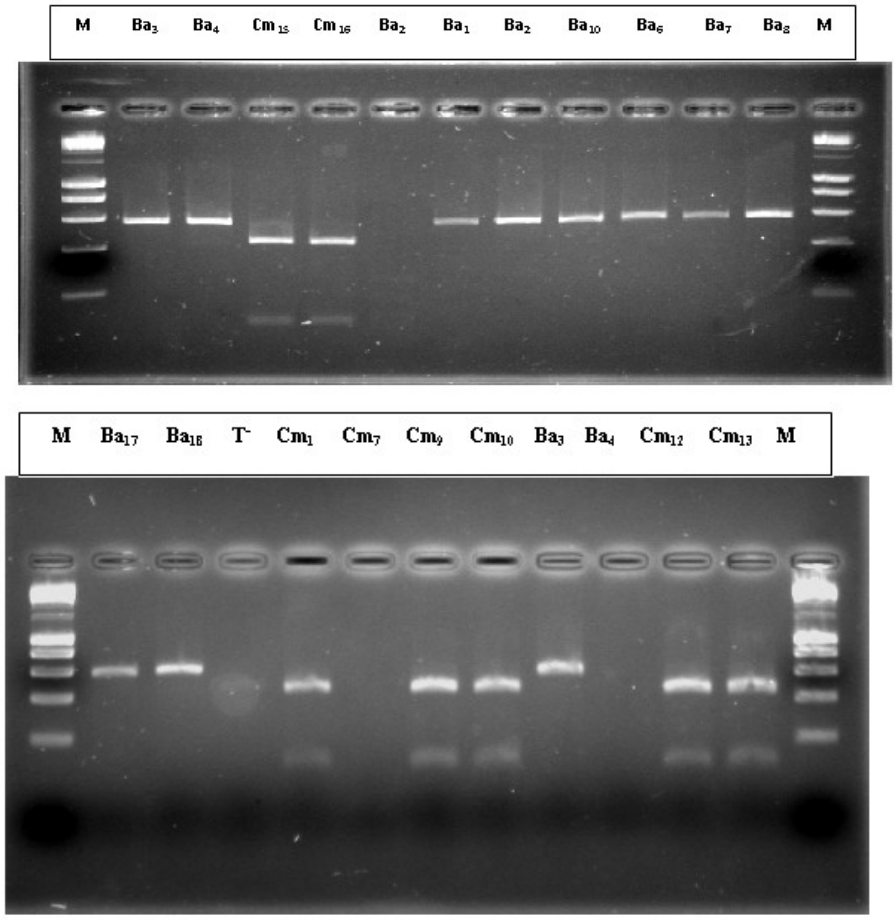

Figure 2: Electrophoretic analysis of polymerase chain reaction (PCR) products of the 600-bp 28S gene digested with *BsmAI* on 2.5 % agarose gel. M = molecular weight marker, Bio 200-bp DNA Ladder. Cm= *C.maculatus,* Ba= *B. atrolineatus* the number next represents the individual.

The entire region amplified by our primers were not found on the sequence download on Genbank for the gene 28S. In fact only one of the attachment sites of our primers was found with Mega4 software. The partial 28S amplified was not only smaller (600 bp for the two species of Bruchidae), but shifted by 261 bps compared to that on Genbank (respectively 724 et 734 pb for *C. maculatus* et *B. atrolineatus*). However, we had 463 bp (*C. maculatus*) and 473 bp (*B. atrolineatus*) common areas between the sequences. In this common portion, no restriction site was found for *B. atrolineatus* but there is one restriction site for *C. maculatus.*

Digestion of *cyt b* gave results that were predicted with no restriction for *B. atrolineatus*, 3 fragments for *C. maculatus*. The bands generated were of the predicted sizes with virtual restriction (Table1). But a different pattern emerges for some individuals who were classified in group of *C. maculatus*. Indeed, the digestion of *cyt b* for these individuals gave 2 fragments instead of the 3. They were supposed to belong to *Acanthoscelides obtectus*, more specific to the common bean, or to *Callosobruchus rhodesianus,* this insect is present in the fields but is poorly adapted to the storage systems.

Table 1—Length of restriction fragments obtained from the polymerase chain reaction products of bruchidea using the *BsmAI* restriction enzymes

Bruchidea species	*Cyt b*	28S
C. maculatus	3 fragments (293pb;129pb; 63 pb)	2 fragments (346 pb;117 pb)
B. atrolineatus	none	None

This difference was not noted with the digestion of 28S, which means the utility of the use of cytochrome b in studies of molecular identification.

The *Cytb* gene has been used in species identification and in taxonomic phylogenetic studies [6]. The *Cytb* gene has been considered one of the most useful genes for phylohenetic work and is probably the best-known mitochondrial gene. Therefore, this gene has been used for a diversity of systematic questions.

Despite the probable existence of another species, a method for rapid identification by PCR-RFLP of two insects that cause the most damage to cowpea in Senegal can be retained. The reaction of digestion by *Bsm*AI enzyme of *Cytb* and 28S allowed us to obtain the patterns specific for each bruchidea specimen.

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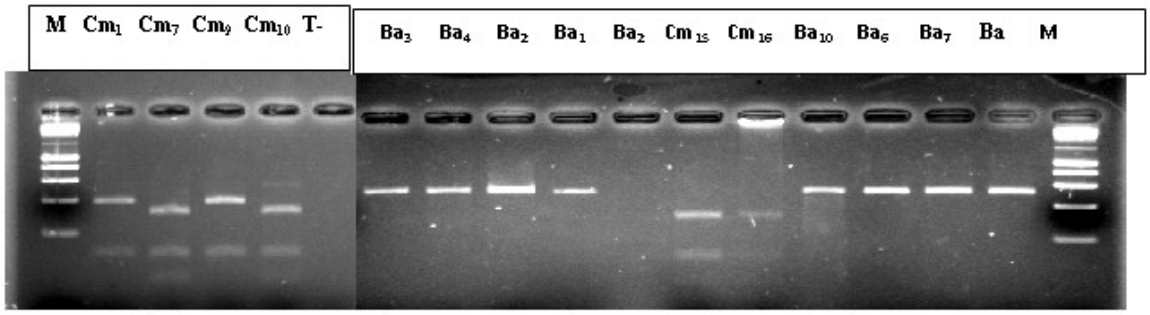

Figure 3: Electrophoretic analysis of polymerase chain reaction (PCR) products of the 600-bp cytochrome *b* gene digested with *BsmAI* on 2.5% agarose gel. M = molecular weight marker, Bio 200-bp DNA Ladder. Cm= *C.maculatus,* Ba= *B. atrolineatus* the number next represents the individual.

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