

Short communication

MOLECULAR EVIDENCE OF *COTESIA FLAVIPES* (CAMERON) (HYMENOPTERA: BRACONIDAE) ESTABLISHMENT IN ETHIOPIA

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ABSTRACT: *Cotesia flavipes* (Cameron) is an Asian origin endo-larval parasitoid of cereal stemborers in the genera of *Chilo*, *Sesamia* and others. It was introduced into Kenya mainly for the control of *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) from India, and North and South Pakistan. After introduction, the parasitoid was released in *C. partellus* prone areas of Eastern and Southern African countries. The establishment rate varies from country to country and region to region within the country. In Ethiopia, the parasitoid was not released, but for the first time recorded in 1999 as identified morphologically. Morphological traits in many instances lead to wrong taxonomic conclusion. Hence, molecular investigation was carried out to confirm whether the parasitoid recorded in Ethiopia is *C. flavipes* or not by running Polymerase Chain Reaction (PCR) of DNA fragments of *C. flavipes* collected from Ethiopia and other African and Asian countries. The PCR analysis using 16S gene (primer) indicated that *C. flavipes* collected from Ethiopia had similar bands with *C. flavipes* from other countries confirming the correctness of the morphological traits used to identify the parasitoid. Hence, the parasitoid recorded in 1999 in Ethiopia was *Cotesia flavipes* which was established without release. To partition the differences that could exist among the different populations of *C. flavipes* and possibly trace the origin of Ethiopian population, advanced molecular techniques such as restricted fragment polymorphism (RFLP) and sequencing will be recommended although the exercises are expensive.

Key words/phrases: 16S, *Cotesia flavipes* populations, DNA, Ethiopia, polymerase chain reaction, sequencing

INTRODUCTION

Cotesia flavipes (Cameron) (Hymenoptera: Braconidae) is an Asian origin endo-larval parasitoid of cereal stemborers in the genera *Chilo*, *Sesamia* and others (Overholt *et al.*, 1997). It was introduced to Kenya mainly for the control of *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) from India, and North and South Pakistan. After introduction the parasitoid was released in *C. partellus* prone areas of Eastern and Southern African countries. The establishment rate varies from country to country and region to region within the country. Both biotic and abiotic factors contribute for differential establishment of *C. flavipes* in Africa. Emana Getu (2005; 2007) and Emana Getu *et al.* (2004) demonstrated that *C. flavipes* population, temperature, relative humidity and the interaction of these factors significantly affect the establishment of the parasitoid. Ethiopia was one of the target countries for the release of *C. flavipes* in the year 2000. However, with the survey conducted in the year 1999 to obtain baseline information for the up coming release, *C. flavipes* was recorded in all major maize and sorghum

growing areas of Ethiopia with varying parasitism rate. The parasitoid collected from Ethiopia was identified as *C. flavipes* by the International Centre of Insect Physiology and Ecology (ICIPE) using morphological traits such as the shape of male genitalia, secuto-scuteller sulcus, rugosity on the propodem and morphometrics (Susan and Overholt, 1997). There are high chances that these non-molecular methods of identification may lead to miss identification which is the common error in insect taxonomy due to factors such as sibling species and geographical influences among others.

Hence, the current experiment was conducted to confirm the parasitoid morphologically named as *Cotesia flavipes* from Ethiopia is the same or different when PCR reaction in 16S gene is used.

MATERIALS AND METHODS

Insect material

Samples of *C. flavipes* were collected from major maize and sorghum growing areas of Ethiopia according to the collection protocol developed earlier by Emana Getu *et al.* (2001). The collected

samples were preserved in 96% Ethanol and taken to Hohenheim University for molecular investigation. During investigation samples from Ethiopia were categorized as Voucher specimen, Northern, Southern, Eastern, Western and mixed populations. For comparison, 30–50 *C. flavipes* individuals were secured from India, North Pakistan, South Pakistan, Kenya and Tanzania through their respective national programs. Each national program sent 30–50 field collected individuals (progenies of 1 cocoon mass) in vials by preserving them in 96% Ethanol to Ethiopia. These were also taken to Hohenheim University together with the samples from Ethiopia for molecular testing and analysis.

Molecular methods

Extraction of DNA

An individual of *C. flavipes* was selected from the working sample and transferred into a 1.5 Eppendorf tube. In the tube the insect was grounded to fine pieces (<0.5 mm in diameter) using first Teflon pestle (disinfected with 70% EtOH) then immersed in liquid nitrogen for few seconds. The fine powder was suspended in 200 µl TES (Tris EDTA solution) buffer and mixed with 2 µl Proteinase K. The mixture was gently mixed using finger tip and heated for 1 hr at 60°C in water bath. After heating, the mixture was mixed using finger tip or manual mixer. Salt concentration was adjusted by adding 56 µl 5M NaCl and 26 µl 10% CTAB (Cetytrimethyl ammonium bromide) solution to the mixture. Then the solution was re-heated for 10 minutes at 60°C in water bath. One volume = 280 µl of chloroform isoamyl alcohol (24:1) was added to the solution in the fume hood by wearing gloves. The solution was gently mixed for five minutes using manual mixer and kept for 30 minutes on ice and centrifuged for 15 minutes at 4°C at 12000 rpm (round per minute). The supernatant was transferred to a new tube and the chloroform discarded in the chloroform bottle in fume hood by wearing gloves. Ammonium acetate at the rate of 45 µl (equivalent to 1/9 by volume) was added to the supernatant and mixed gently and kept on ice for 30 minute. After centrifugation for 20 minutes at 4°C at 12000 rpm the upper phase was transferred to a new tube and 300 µl isopropanol was added and stored at 4°C for overnight. The solution was centrifuged at 4°C for 20 minutes at 12000 rpm to precipitate the DNA. Then the supernatant was carefully discarded and the pellet was washed twice with cold (stored at 4°C) 70% ethanol and dried at ambient temperature. The

pellet was re-suspended in 100 TE-buffer for 4–5 hours on ice. The product (DNA) was kept in refrigerator at 4°C until use.

Characteristics and sequences of the 16S

The 16S forward gene region is a mitochondria DNA (mtDNA) having a sequence of 5'-CACCTGTTTATCAAAAACAT-3' (Dowton and Austin, 1994), while the 16S reverse gene region is an mtDNA having 5'-CTTATTCAACATCGAGGTC_3' sequence (Whitefield, 1997).

PCR technique and profiles

A 25 reaction mixtures BSA (Bovine serum albumin) (0.5 µl), MCl₂ (2.0 µl), 10X PCR buffer ((NH₄)₂SO₄) (2.5 µl), 2mM DNTPs (2.5 µl), Primer forward 16Sf (1 µl), Primer reverse 16Sr (1 µl), DNA Tag (green) polymerase (0.2 µl), H₂O (10.3 µl) and DNA template (5 µl) were prepared for each sample. The mixtures were loaded on to the PCR machines according to the recommended profiles (Singh, 2005).

The PCR profiles for amplification of 16S were initial denaturation at 94°C for 3 minutes, denaturation at 94°C for half a minute, annealing at 50°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 7 minutes.

Electrophoresis

Analysis of the PCR product was made on Agarose TBE gel electrophoresis. About 1.2 g Agarose serva (1.2%) was mixed with 100 ml of 1X TBE and then cooked in microwave at 700 watts for 2-3 minutes. The mixture was allowed to cool for 3 to 5 minutes and poured on the gel plate in the centre and a comb with 14 pegs was inserted to make a well. The comb was carefully removed without damaging the wells. Buffer (1X TBE) was poured to cover all the field of the gel plate. From each sample 5 µl PCR product and 2 µl loading dye were mixed and centrifuged for two seconds for complete mix up. The mixture (6.5 µl) of each sample was poured into the wells in the gel using pipette. As size marker 5 µl 100 bp was used. The gel plate was covered and fitted to electric line to run electrophoresis at 80 voltage for 1 hr. After 1 hr the gel was stained for 15–20 minutes in 1% Ethidium bromide and then the stain was washed for 10–15 minutes in water bath and photographed under a special camera connected to the computer. Molecular methods were repeated for 10 times *i.e.*, 10 individual insects were used from each sample for molecular analysis.

RESULTS AND DISCUSSION

The PCR analyses for all replications indicated that all *Cotesia flavipes* populations from Ethiopia had similar bands with *Cotesia flavipes* population from the mother stocks (*Cotesia flavipes* from India, North and South Pakistan) and the introduced populations to Africa (Kenya and Tanzania) (Fig. 1).

Cotesia flavipes is the most popular bio-control agent used against cereal stemborers on several crops such as maize, sorghum, sugar cane and others in Asia and Africa mainland and Islands with great success (Overholt *et al.*, 1997; Singh, 2005). The problem of *C. partellus* in coastal Kenya before the introduction of *C. flavipes* was very immense resulting in crop failure in some years and seasons (Overholt *et al.*, 1997). However, the

release of *C. flavipes* since 1993 was highly suppressed the density of *C. partellus* which also impacted yield loss due to the pest. Similar impacts of *C. flavipes* in Uganda, Tanzania and South African countries such as Zambia, Zimbabwe and Malawi were reported (Zhou *et al.*, 2001).

In Ethiopia, the parasitoid voluntarily established without release and recorded from all major sorghum and maize growing areas of the country in 1999 (Emana Getu *et al.*, 2001; 2004). In 1999 the parasitism rate of the parasitoid was 7.5%, whereas the 2005/2006 survey showed that the parasitism rate reached 58% indicating the fast population growth of *C. flavipes* since its arrival in Ethiopia (Emana Getu *et al.*, 2001; Emana Getu, 2005).

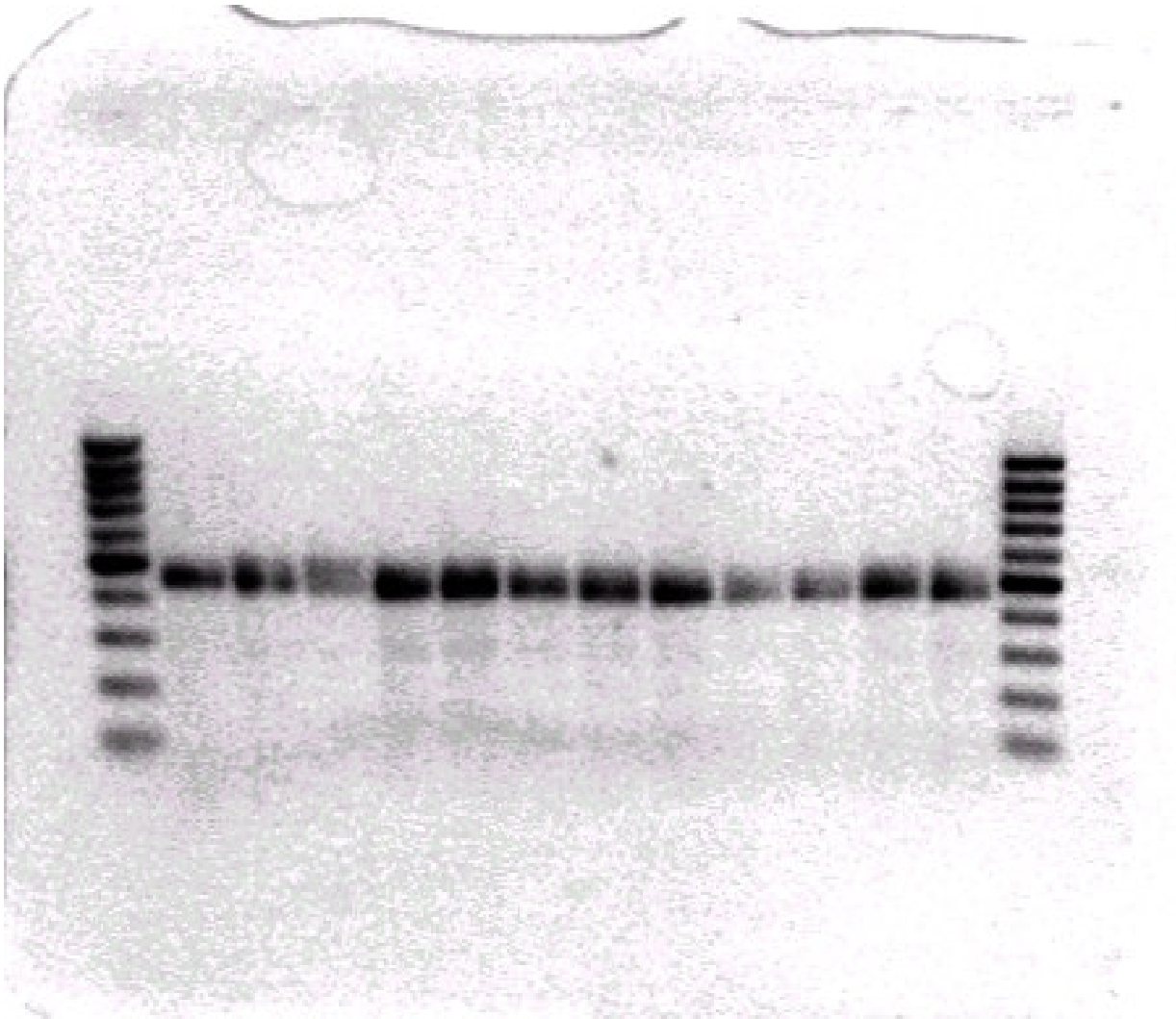


Fig. 1. Gel electrophoresis of amplified 16S gene PCR products [Lane 1 and 14 = 100 bp; Lane 2 = Ethiopian population (voucher specimen); Lane 3 = North Pakistan population; Lane 4 = Kenya population; Lane 5 = Tanzania population; Lane 6 = India population; Lane 7 = South Pakistan population; Lane 8 = Ethiopia population (Central); Lane 9 = Ethiopia population (Eastern); Lane 10 = Ethiopia population (Southern); Lane 11 = Ethiopia population (Northern); Lane 12 = Ethiopia population (Western); Lane 13 = Ethiopia population (Mixed)] (Similar bands were observed in 10 replications).

The source of *C. flavipes* recorded in Ethiopia was speculated to be the Somalia release of 1997 near Shebele River bordering Ethiopia as the parasitism rate in Ethiopia towards the direction of the release site in Somalia was relatively higher. If this speculation is true *C. flavipes* spread 2000 km away from the point of release in Somalia to Ethiopia within three years indicating a very high success of the parasitoid in the country may be due to very conducive ecological factors existing in the country (Emana Getu *et al.*, 2001).

At the moment *C. flavipes* exist in Ethiopia under natural condition, but it has to be considered in the future biological control program of cereal stem borers in the country. For operating biological control program, the taxonomy of the parasitoid considered in the program is very crucial (Driesche and Bellows, 1996). Downton and Austin (1994), and Reineke *et al.* (1999) recommended the use of molecular methods such as PCR for such kind of work as the methods are the finest method of systematics with 2% level of significance. The current molecular analysis of different populations of *C. flavipes* confirmed that the earlier identification using morphological characters is correct since all *C. flavipes* collected from Ethiopia are identical with the mother stocks from India, North and South Pakistan, and introduced populations.

Even though differences among Ethiopian populations of *C. flavipes* is not expected at the moment, to partition the possible differences that the Ethiopian population has with the Asian and other African populations, and possibly trace the origin of Ethiopian population, molecular testing and analyses should be done using RFLP and DNA Sequencing.

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