A PCR–RFLP tool for differentiating *Helicoverpa armigera* and *H. assulta* (Lepidoptera, Noctuidae)

The family Noctuidae includes some of the most damaging agricultural pests worldwide. In India, two species of *Helicoverpa* and one species of *Heliothis* have been recorded, viz. *Helicoverpa armigera* (Hubner), *Helicoverpa assulta* Guenee and *Heliothis peltigera* (Denis and Schiffermuller)[1]. The three species were originally included under the same genus *Heliothis* before *armigera* and *assulta* were transferred to the new genus *Helicoverpa*[1–3]. Here we report a molecular tool for distinguishing the two species of *Helicoverpa.*

H. armigera and *H. assulta* are found throughout Africa, Asia, parts of Australasia and the South Pacific. *H. armigera* is polyphagous[2,4] and insecticide-resistant[5]. *H. assulta* is an oligophagous pest. Its principal hosts are tobacco, chilli bell pepper and wild hosts in the genus *Datura*[1,2,6]. There are no reports of control failures or insecticide resistance in this species in the Indian subcontinent. However, control failures of *H. assulta* on capsicum have been attributed to insecticide resistance in South Korea[6].

During field collections of *H. armigera* eggs and larvae in India, it was common to come across mixed populations of *H. armigera* and *H. assulta* on tobacco and on wild hosts in the family Solanaceae. Between April and June, mixed populations of *H. armigera* and *H. assulta* occurred on Datura and other solanaceous host plants like capsicum and chilli. Light traps operating between August 1974 and May 1987 at ICRISAT, Patancheru, indicated that *H. armigera* was at least 100 times more abundant than *H. assulta* over most of the cropping season[1,2]. Peak catches of *H. assulta* were confined from August to October, which also coincided with those of *H. armigera* on cotton. In view of these overlapping populations, it may possibly be confusing to distinguish between *H. armigera* and *H. assulta*. *H. assulta* is considered a minor pest, but its importance may be undermined because of the similarity of both larvae and moths to those of *H. armigera*[7]. It requires the services of a trained entomologist to differentiate the two species in the adult stage, applying taxonomic keys as described by Hardwick[3] and Mathews[8,9]. Eggs and neonates are virtually indistinguishable in mixed populations. A molecular, stage-independent tool based on the mitochondrial genome is being proposed for distinguishing the two species of *Helicoverpa.*

H. assulta larvae were collected from *Datura* in and around cotton fields of Nagpur. *H. armigera* were collected as eggs from cotton. Larvae were reared till pupation on semi-synthetic diet[10]. The emerging moths were identified using the taxonomic key described earlier[3,8,9]. Genomic DNA was isolated from the thorax of female moths using the protocol described by Zhang *et al.*[11]. The mid CO-1 (cytochrome oxidase) region has a high functional significance and was therefore chosen for the study. Primers were designed to amplify the specific CO-1 region[12], C1-J-2090 and C1-N-2659, whose sequences are 5′-AGT TTT AGC AGG AGC AAT TAC TAT-3′ and 5′-GCT AAT CCA GTA AAT AAA GG-3′ respectively. A PCR reaction in a volume of 25 µl was programmed as follows: 94°C for 2 min, 94°C for 45 s, an annealing temperature of 50°C for 45 s, 72°C for 1.3 s, in 38 cycles, 72°C for 10 min and 4°C to end the reaction. The amplified fragment was subjected to sequencing on capillary-based CEQ 2000XL DNA Analysis System (Beckman Coulter, Inc, USA) at the Central Institute for Cotton Research, Nagpur and the sequences were submitted to National Centre for Biotechnology Information (NCBI) Genebank (Accession numbers are AY 264944, AY 264943). The sequence was subjected to a search (Premier Primer 5.0, Premier Biosoft International, CA, USA) for the identification of unique restriction sites specific to one species in the sequenced region with respect to the other. One of the unique restriction enzymes (RE) *Rsa*I, which cuts at 5′-GT↓AC-3′, was obtained from Q Biogene (India). The PCR product was directly digested at 37°C for 6 h with *Rsa*I in a 20 µl reaction containing 2 U restriction enzyme, 2 µl 10X RE buffer and distilled water. Digested DNA bands were separated on 2% agarose and were visualized on Kodak EDAS 230 gel documentation system.

The primers used in the study were designed to amplify a 598 bp fragment corresponding to mid-to-near terminal region of CO-1. Sequence of the amplified fragment was subjected to multiple sequence alignment using Clustal X[13]. Nucleotide sequence and translated amino acid sequences, with the invertebrate mitochondrial genetic code[14] in each of the strains, resulted in high level of consensus between the two *Helicoverpa* species.

Mutations in the sequenced region were observed between *H. assulta* and *H. armigera*, whose significance is not being speculated herein. The region sequenced in this study was capable of being selectively restricted in *H. armigera* with eight restriction enzymes, with *Rsa*I being one of them. *Rsa*I was chosen for the study as it demonstrated the ability of cutting the sequenced region approximately in the centre, resulting in two fragments of approximately 333 and 265 bp each, as shown in Figure 1. Its recognition site is masked by a mutation GT↓AC in *H. armigera* that is replaced with GT↓TC in *H. assulta*, which is responsible for the absence of restriction digestion.

PCR–RFLP as a tool demonstrates reliable differentiation of the two species. It offers support to conventional taxonomic differentiation based on morphological features. Both techniques, however, require skill and expertise. Mutilated museum specimens that are difficult to study using the taxonomic key can be readily identified with this tool. This method can be used as a molecular tool for distinguishing *H. armigera* and *H. assulta* from each other especially in ecosystems that harbour a mixture of both species. Species

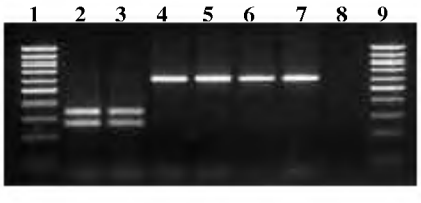

Figure 1. Restriction digestion of PCR-amplified partial CO-I region of *H. armigera* and *H. assulta* with *Rsa*I. Lanes 1, 9, 100 bp MW ladder; lanes 2, 3, *H. armigera* PCR product digested with *Rsa*I; lane 4, *H. armigera* PCR product undigested control; lanes 5, 6, *H. assulta* PCR product digested with *Rsa*I, lane 7, *H. assulta* PCR product undigested control; lane 8, Negative control.

identification in *Helicoverpa* would significantly influence the choice of pest management strategies, considering that *H. armigera* has become insecticide-resistant, while *H. assulta* continues to be susceptible.

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Regeneration of plants from root explant of two Indian cultivars of *Brassica campestris* L. through somatic embryogenesis

Brassica campestris L. is cultivated worldwide as an important edible and industrial oilseed crop. Though organogenic shoot regeneration from callus[1,2], leaf disc[3], cotyledon[4–11], cotyledonary petiole[12,13], hypocotyl[14,15], isolated protoplasts[16,17], anther[18] and isolated microspore[19–21] of *Brassica campestris* L. has been reported, regeneration through embryogenesis has not yet been reported in this species. Plant regeneration from root explant in Cruciferae has been reported in *B. oleracea*[22] and *Arabidopsis thaliana*[23–25], but not from *B. campestris*[26]. Embryogenic plant is considered to be most desirable for *Agrobacterium*-mediated transgenic plant production. The present correspondence describes efficient plant regeneration protocol through embryogenesis from root explants of two Indian cultivars of *B. campestris*, i.e. cv. B-54 (Agrani) and cv. B-9 (Benoy). Henceforth the two cultivars will be designated as B-54 and B-9 respectively, in the text.

Medium – BS1: Half concentration of MS salts and vitamins[27], 10 g l^{-1} sucrose, 0.8% agar, pH 5.8; BS2 to BS4: As Marton and Browse[24], but hormone requirement of respective medium is depicted in Table 1; MS-H: MS salts and vitamins, 20 g l^{-1} sucrose, pH 5.8, 1% agar. All the media were supplemented with 3 ml l^{-1} Miller's solution[24]. In two other experiments, either 2.5 mg l^{-1} silver thiosulphate or 3.3 mg l^{-1} $AgNO_3$ was used. All hormones, silver thiosulphate and $AgNO_3$ were filter-sterilized and added to autoclaved media before plating. Silver thiosulphate or $AgNO_3$ was not added to MS-H medium.

Surface-sterilized seeds (30 min in liquid detergent Teepol, washed in tap water, 5 min in 0.2% $HgCl_2$ solution, washed in sterile water six times) were germinated on BS1 medium in petri dishes (90 mm Ø) which were maintained at 26 ± 1°C in the dark for two days followed by four days incubation in a 16/8 h light/dark photoperiod (light intensity 3000 lx), till the roots were 5–6 cm in length. One set of petri dishes was maintained in the dark at 26 ± 1°C and the other set in the dark at 11 ± 1°C for cold treatment for 7–21 days.

For each root culture experiment, ca. 2–10 mm long taproot segments from apical (towards root apex), basal (towards root–hypocotyl transition) and middle regions were excised separately from 7 to 21-day-old seedlings and wounded by squeezing firmly with fine forceps at 2–5 mm intervals and spread uniformly (25–30 segments/90 mm Ø plate) over the embryo induction medium (BS2); and cultured at 26 ± 1°C in a 16/8 h light/dark photoperiod (light intensity 3000 lx) for 7–14 days. Subsequently, the explants were transferred to the embryo elongation medium (BS3) and cultured for another 10–14 days. The embryogenic root segments were transferred to embryo maturation medium (BS4). After the visible appearance of shoots in these embryos, they were transferred to MS-H medium for rooting and further development. The rooted plants were hardened and transferred to field, according to Mandal and Sikdar[28].

Table 1 depicts the high responsive media used in different stages of embryo development. In the embryo induction phase, BS2 III and BS2 XII gave the best response for B-54 (88.2 %) and B-9 (90.8%) cultivars respectively (Table 2). In the elongation phase BS3-type medium, i.e. BS3 II, III, VI and IX for B-54 and BS3 XVII and XVIII for B-9 could show high response in developing green embryos (0–12/root segment, average 3.52 for B-54