DNA identification of *Busseola* (Lepidoptera: Noctuidae) larvae in Ethiopian sugarcane

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Noctuidae is one of the largest lepidopteran families, encompassing about 20,000 species (Holloway 1998). Some 157 described species of Noctuidae are known to be cereal stem borers in the Afrotropical region (Moyal 2006) and the most economically important of these belong to the genera *Busseola* and *Sesamia*. Accurate identification of pest species is the first and most fundamental step to developing sound pest management strategies (Szalanski et al. 2003). However, many of these closely related stem borers are difficult to distinguish from each other morphologically, and no key is available to cover all noctuid stem borers (Holloway 1998).

Misidentifications of stem borers have occurred frequently, resulting in the publication of misleading data that are perpetuated, often for decades (Polaszek 1992). This problem is pronounced when identification of larvae is considered. Morphological structures such as setae are easily broken and frequently missing from alcohol-preserved and deep-frozen material. This makes identification even more difficult and results can be unreliable (Meijerman & Ulenberg 1998). Moreover, even when all of the setae are present, differences between species at the larval stage are minor and often it is not possible to identify larvae to species level. For instance, in the case of noctuid stem borers, species belonging to different genera, e.g. *Busseola fusca* Fuller and *Sesamia calamistis* Hampson, can have exactly the same long and microscopic setae, and the only difference in larval morphology is a slight change in the position of two setae on one segment (Moyal & Tran 1989). Distinction of species within these genera is generally more difficult and is usually not possible.

Hebert *et al.* (2003) proposed that the analysis of sequence diversity in *cytochrome-c oxidase I* (COI) gene of the mitochondrial DNA could serve as the core of a global identification system in the animal kingdom (‘DNA barcoding’). The potential of DNA barcoding has been demonstrated in many recent studies, reviewed by Vogler & Monaghan (2007) and Waugh (2007). Although this approach is not without controversy (Cognato 2004; Will *et al.* 2005; Brower 2006), the lack of adequate morphological taxonomic services makes the molecular approach an attractive alternative. The use of DNA-based technologies has been suggested as a good option to solve the current problems in identification of field-collected material (Hogg & Hebert 2004; Janzen *et al.* 2005; Hajibabaei *et al.* 2006). However, these papers did not adequately address the important question of whether the COI gene has the discriminatory power to correctly identify closely related species (Will *et al.* 2005). In this paper, we demonstrate the utility of DNA barcodes for identification of Ethiopian *Busseola* species.

*Busseola* larvae were collected from sugarcane and wild host plants bordering sugarcane fields of small-scale farms and commercial estates in Ethiopia (Table 1) on two occasions, November 2003 and February 2004. Specimens were placed into 30 ml plastic vials each containing a piece of sugarcane stalk or artificial diet in which to complete their development. However, none of the collected specimens developed to adult stage. The dead larvae were taken out of the stalks and/or artificial diet and kept in 95% ethyl alcohol in sealed screw-top 30 ml glass vials.

Identification of adult *Busseola* specimens was carried out by comparison of male and female genitalia with those of the type specimens deposited in the Natural History Museum (BMNH, London). *Busseola fusca* and *Busseola phaia* Bowden

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are easily distinguished by their genitalia. In *B. phaia* males the clasper is much smaller than in *B. fusca*. The costal expansion is more ridge-like in *B. phaia* and produced into a large spine in *B. fusca*. The female genitalia are also quite different, with *B. phaia* having only the plate posterior to the ostium sclerotized while *B. fusca* shows strong sclerotization of both anterior and posterior plates.

Genomic DNA was extracted from individual larval thoraxes using the Qiagen DNeasy™ Tissue Kit as recommended for animal tissues, and the extracted DNA was stored at –20 °C until required for amplification. Voucher specimens are housed at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, KwaZulu-Natal, South Africa.

Polymerase chain reaction (PCR) amplification was performed in a 50 µl volume containing 1X PCR buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 15 pmol of each PCR primer, 1 unit of SuperTherm Gold Taq DNA polymerase (JMR Holdings, Kent, United Kingdom) and 1 µl of genomic DNA. Primers used in the study were: LCO1490 (5’-GGTCAACAAATCATAAAGATATTGG-3’) and HCO2198 (5’-TAAACTTCAGGGTGACCAAAAAATCA-3’) from Folmer *et al.* (1994). PCR was performed using a Perkin Elmer GeneAmp PCR System 2400 (Applied Biosystems), under the following conditions: 94 °C for 11 min, 30 cycles of (94 °C for 30 s, 50–55 °C for 30 s, 72 °C for 30–90 s), 72 °C for 7 min, 4 °C hold. Amplified DNA was purified using the Qiagen QIAquick™ PCR purification kit following the manufacturer’s protocol. DNA sequencing reactions were performed using the ABI PRISM® BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems), cleaned using Ethanol/EDTA precipitation with slight modification of the manufacturer’s protocol, and sequences were visualized on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, U.S.A.). Editing and assembling DNA sequence chromatograms was completed using the Staden package (Staden 1996). Sequences were then aligned by hand using BioEdit (Hall 1999). The *Sesamia nonagrioides* Lef. (Lepidoptera: Noctuidae) sequence was downloaded from GenBank (accession number AY649322) and used as an outgroup. Phylogenetic analysis was performed by Maximum Parsimony (MP) using an exhaustive search in PAUP* v4.0b10 (Swofford 1998). Tree reliability was assessed by bootstrap analysis with 1000 replications using branch-and-bound searches.

These DNA sequences were then sent to the fourth author for comparison with sequences from adult insects collected in Kenya, Uganda and Cameroon (Table 2) for a phylogenetic study of African noctuid stem borers that is presently underway (P.M., unpubl.).

A phylogenetic analysis of the 658 bp COI sequences produced for the Ethiopian *Busseola* specimens separated the specimens into two groups. Group 1 contained four haplotypes (415, 450, 715 and 716; Fig. 1) differing from one another by 2–5 base-pairs (i.e. 0.32–0.77 % divergence). Group 2 contained two haplotypes (451/452 containing sequence 451 and 452 and 717; Fig. 1) differing from one another by one base pair (i.e., 0.2 % divergence). Groups 1 and 2 differed from one another by an average of 33 base-pairs (5.02 % divergence). This result suggested that two species of *Busseola* were present in our sample. These DNA sequences were sent to the fourth author to determine if they could be matched with his overlapping COI sequences of *Busseola* species.

The last 343 bp of the 658 bp sequences from larvae collected in Ethiopia (Table 1) were aligned with the first 343 bp of the sequences from identified adult *B. fusca* and *B. phaia* (Table 2). This 343 bp

<table>
<thead>
<tr>
<th>DNA No</th>
<th>GenBank Acc. No.</th>
<th>Farmtype</th>
<th>Location</th>
<th>Longitude, Latitude</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>415</td>
<td>DQ337201</td>
<td>Estate</td>
<td>Wonji</td>
<td>39°12’E 08°31’N</td>
<td><em>Cyperus dives</em></td>
</tr>
<tr>
<td>450</td>
<td>DQ337199</td>
<td>Small-scale</td>
<td>Inguti</td>
<td>37°06’E 11°24’N</td>
<td>Sugarcane</td>
</tr>
<tr>
<td>451</td>
<td>DQ337196</td>
<td>Small-scale</td>
<td>Goma</td>
<td>36°36’E 07°51’N</td>
<td>Sugarcane</td>
</tr>
<tr>
<td>452</td>
<td>DQ337195</td>
<td>Small-scale</td>
<td>Sidama</td>
<td>38°26’E 06°54’N</td>
<td>Sugarcane</td>
</tr>
<tr>
<td>715</td>
<td>DQ337200</td>
<td>Small-scale</td>
<td>Mankusa</td>
<td>37°11’E 10°40’N</td>
<td>Sugarcane</td>
</tr>
<tr>
<td>716</td>
<td>DQ337198</td>
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<td>Mankusa</td>
<td>37°11’E 10°40’N</td>
<td><em>Pennisetum purpureum</em></td>
</tr>
<tr>
<td>717</td>
<td>DQ337197</td>
<td>Small-scale</td>
<td>Goma</td>
<td>36°36’E 07°51’N</td>
<td>Sugarcane</td>
</tr>
</tbody>
</table>

Table 1. Collection area data for larval Ethiopian *Busseola* spp.
region of overlap was analysed as a separate dataset. Sequences of the two groups (Fig. 1) matched exactly the sequences obtained from two species collected in East Africa: B. fusca and B. phaia (Fig. 2). Three sequences (451, 452 and 717) belonged to the same group as an identified B. phaia from Uganda (p-distance = 0) (GenBank accession number DQ663577), and specimen 715 from Ethiopia belonged to the same haplotype as two identified B. fusca from Kenya (GenBank accession numbers DQ663573 and DQ663574), whereas the other specimen (450) differed by 0.3% (one difference in 343 nucleotides).

DNA sequence analysis of the COI fragment of mitochondrial DNA was successful in discriminating between two species of Busseola in Ethiopia. Levels of divergence within species were less than 1%, whereas inter-species divergence exceeded 4.5%. Similar levels of divergence have been reported in other studies of lepidopterans (Cognato 2004; Sezonlin et al. 2006).

DNA-based methods in this case were found to be a quick, easy and reliable method for identification of species. This method provides data that could be used to develop quicker and more cost effective methods such as PCR-RFLP and multi-
plex-PCR. The resulting methods may then be solutions for conditions in Africa where there is an acute shortage of experts and rearing facilities to keep field-collected insects alive until emergence of adults for morphological identification.

The use of barcoding as a taxonomic tool has been criticized by many authors (e.g. Lipscomb et al. 2003; Lee 2004; Will & Rubinoff 2004), and it can result in misidentifications, particularly of closely related species, if there is not adequate sampling of species from across their geographic range when the reference data set is collected. However, the method was successful in the identification of the Ethiopian *Busseola* species. This is in large part due to a comprehensive study of the ecology, morphology and molecular systematics of African noctuid stem borers presently being led by Institut de Recherche pour Développement (IRD). That study has provided sufficient data on intraspecific and interspecific variability to allow us to make confident species determinations. DNA barcoding will be reliable in noctuid African stem borers only when we have sufficient sampling of the species from different countries. On the other hand, the COI data from the seven larvae immediately suggested to us that we had collected two different species in our field surveys, although this was not apparent using morphology. Although not conclusive by itself, the barcode data led us to seek further corroborating evidence, demonstrating the heuristic value of the DNA barcoding approach.

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