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Phylogeography Based on Mitochondrial DNA Reveals Large Genetic Differences within Eswatini's *Eldana saccharina* Populations

Mphumelelo Ndlovu[1], Sibusiso Sibandze[2], Bonginkosi Raymond Dlamini[3], Yoseph Assefa[4]

[1]Eswatini Sugar Association (ESA), Technical Services, Kingdom of Eswatini
[2]National Maize Corporation, Farmer Development Unit, Kingdom of Eswatini
[3]His Majesty's Correctional Services, Crop Production Unit, Kingdom of Eswatini
[4]University of Eswatini (UNESWA), Luyengo Campus, Kingdom of Eswatini
mphumelelo@esa.co.sz; sibucomfort@gmail.com

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Abstract

The African sugarcane stalk borer, *Eldana saccharina*, is a widespread pest across sub-Saharan Africa and often exhibits substantial genetic structuring across its broad distribution, yet little is known about its genetic variation within the relatively small nation of Eswatini. This study employed mitochondrial cytochrome c oxidase subunit I (COI) gene sequencing to investigate the phylogeography of *E. saccharina* in sugarcane-producing areas of Eswatini. Analysis of 69 specimens revealed two distinct genetic groups with significant genetic differentiation, despite the limited geographic scale of sampling. A minimum spanning network and a maximum parsimony tree clearly demonstrated the separation of the population into two discrete lineages. The observed genetic differences are likely shaped by the pest's specialization on different host plants and historical biogeographic processes. This work provides the first DNA-based characterization of *E. saccharina* in

Eswatini and the first report on the phylogeography of its populations in the country. The dataset generated constitutes a valuable addition to existing *E. saccharina* taxonomic resources and will support more rapid and informed decision making in the design and implementation of biological control programs in Eswatini and across the African continent.

Keywords: Cytochrome c Oxidase Subunit I; *Eldana saccharina*; Eswatini; Phylogeography; Sugarcane.

Introduction

Eldana saccharina Walker (Lepidoptera: Pyralidae), commonly known as the African sugarcane stem borer, is an indigenous African insect (Assefa, 2006). It is a polyphagous pest, feeding on cultivated graminaceous crops as well as various wild grasses and sedges (Conlong, 1994). The first documented instance of *E. saccharina* infesting sugarcane was in West Africa in 1864 (Walker, 1865). Subsequently, it was recorded in maize and sugarcane in Eastern and Southern Africa (Dick, 1945; Girling, 1972). While *E. saccharina* has been reported in maize and sorghum in Southern Africa, it rarely causes significant crop damage in this region (Atkinson, 1980). This contrasts sharply with West Africa, where it is a major pest of maize and sorghum (Shanower *et al*., 1993).

Studies have revealed that *E. saccharina* exhibits significant phenotypic and behavioral variation across its range. This includes differential responses to control agents and distinct host plant preferences in different parts of Africa (Conlong, 2001). The species' complex behavioral patterns and diverse natural enemy guilds, coupled with a lack of corresponding morphological diversity, make it an ideal candidate for molecular systematic analysis (Scheffer, 2000). Given the increasing economic importance of *E. saccharina* (Mahlanza *et al*., 2014), there is a pressing need for effective control strategies. A fundamental prerequisite for informed decisions on biological control, particularly the selection of natural enemies and for the correct interpretation of ecological investigations is to determine the degree of relatedness among different populations. Such information is also crucial for developing habitat management control options.

Understanding the quantification and distribution of genetic variability, as well as the historical and structural aspects of populations, is fundamental for making informed decisions in various ecological and management contexts (Balkenhol *et al*., 2017). This

knowledge is particularly critical for the selection of effective natural enemies for biological control, the implementation of improved management strategies, and the accurate interpretation of ecological investigations. *Eldana saccharina* Walker (Lepidoptera: Pyralidae) exhibits significant behavioral variation across its range. However, it remains unclear whether these behavioral differences correlate with underlying genetic distinctions. Extensive molecular data are therefore required to better understand the genetic basis of these behavioral patterns.

Phylogeographic studies on insect species in Africa are becoming more common (e.g., Assefa *et al.,* 2006; Goftishu *et al.*, 2016; Goftishu *et al.*, 2019; Hlaka *et al.*, 2021). Given the widespread distribution of *E. saccharina* across the African continent, an analysis of its population structure can provide valuable insights into its colonization routes and the barriers that may have impeded its dispersal. The pan-African distribution and suspected existence of different biotypes underscore the need for a comprehensive genetic analysis of this species. The primary objective of this project was to characterize the genotypic diversity of *E. saccharina* populations. We collected samples from sugarcane fields in three major estates in Eswatini (Simunye, Ubombo, and Mhlume) and from small-scale farmers' fields. The study also aimed to generate ecological information and develop molecular diagnostic methods to support the creation of a robust biological control program for managing this pest and to facilitate the efficient use of its natural enemies.

Materials and Methods

Survey Methodology

Surveys were conducted from March to June 2024 to collect samples of the pest *Eldana saccharina*. Samples were gathered from twelve sugarcane grower fields across the country (as detailed in Table 1 and illustrated in Fig. 1). In every field visited, the geographic coordinates of the locality were recorded using a GARMIN 12X portable Geographic Positioning System (GPS).

Sample Collection and Preservation

Individual specimens of *E. saccharina* were placed into small glass vials containing absolute alcohol for preservation. The vials were then sealed and labelled with all the necessary and relevant information pertaining to the collected samples.

Molecular Analysis of *Eldana saccharina*

DNA Processing and Amplification

Representative *Eldana saccharina* specimens from the surveyed sugarcane fields were selected for subsequent molecular analysis. Tissue samples from these specimens were sent to Inqaba Biotechnological Industries in Pretoria, South Africa, for DNA extraction, Polymerase Chain Reaction (PCR) amplification, and sequencing.

The primer pair LCO1490 and HCO2198 (5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994) was employed to amplify a fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene.

Sequence Editing and Phylogenetic Analysis

DNA sequences were initially edited and assembled using the Staden package (Staden 1996). Sequences were then aligned using ClustalX (Thompson et al. 1997) and manually corrected with the BioEdit 5.0.9 sequence alignment editor (Hall 1999) to yield a final dataset of 69 sequences with a length of 603 base pairs (bp).

Phylogenetic Reconstruction

Pairwise distances were calculated using the Kimura-2-parameter (K2P) model (Kimura, 1980). A phylogenetic tree was reconstructed using the Neighbour-Joining method (Saitou & Nei, 1987). Clade support was estimated with 10,000 bootstrap replicates (Felsenstein 1985). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are expressed as the number of base substitutions per site. All evolutionary analyses were performed using the MEGA11 software (Tamura et al., 2021). *Galleria mellonella* (L.) was used as an outgroup due to its close phylogenetic relationship, belonging to the same subfamily (Galleriinae) as *E. saccharina*.

Haplotype Network

A haplotype network was generated using PopART software with the Median-joining algorithm (Bandelt et al., 1999) to determine the genealogical relationships among the *E. saccharina* populations.

Table 1. Sugarcane growers from where specimens of *Eldana saccharina* were collected.

DNA No and Growers name	Total No. of Sequences	Location	Administrative regions	Haplotype name
28_EtF 29_EtF 31_MaF 59_UmE 36_USL 37_USL 40_EnF 41_EnF 45_PhF 72_MSE 73_MSE 6_UnP 10_UnP 12_UnP 13_UnP 14_UnP 17_UnP 18_UnP 21_UnP 32_MaF 35_MaF 38_USL 39_USL 42_EnF 43_RSS 44_RSS 49_CrP 52_CrP 54_CrP 60_MSE 61_MSE 64_MSE 69_MSE 71_MSE	34	Sidvokodvo, Lavumisa, Simunye, Big-Bend, Mhlume	Lubombo, Manzini, Shiselweni	Haplotype_1
30_EtF 8_UnP 15_UnP 20_UnP 55_SiF 62_MSE 65_MSE	7	Sidvokodvo, Big-Bend, Mhlume,	Lubombo, Manzini	Haplotype_2
53_CrP 46_PhF 47_DMF 4_UnP 9_UnP 11_UnP 16_UnP 23_UnP 25_UnP 27_UnP 48_DMF 67_MSE 70_MSE 2_UnP	14	Big-Bend, Mhlume	Lubombo	Haplotype_3
33_MaF 34_MaF 50_CrP 63_MSE 19_UnP	5	Lavumisa, Big-Bend, Mhlume	Lubombo, Shiselweni	Haplotype_4
68_MSE 5_UnP 7_UnP	3	Mhlume, Big-Bend	Lubombo	Haplotype_5
22_UnP 26_UnP	2	Big-Bend	Lubombo	Haplotype_6
24_UnP	1	Big-Bend	Lubombo	Haplotype_7
51_CrP	1	Big-Bend	Lubombo	Haplotype_8
57_UmE	1	Simunye	Lubombo	Haplotype_9
58_UmE	1	Simunye	Lubombo	Haplotype_10
1_UnP	1	Big-Bend	Lubombo	Haplotype_11

NB: EtF-Etibusisweni Farm, MaF-Maplotini Farm, UmE-Umbuluzi Estate, USL-Ubombo Sugar Limited, EnF-Enthuthwini Farm, UnP-United Plantations, CrP-Crookes Plantations, RSS-Royal Swazi Sugar Cooperation, DMF- Daniel Mkhonta Farm, PhF-Phendukani Farm, SiF- Sivunga Farm, MSE- Mhlume Sugar Estste

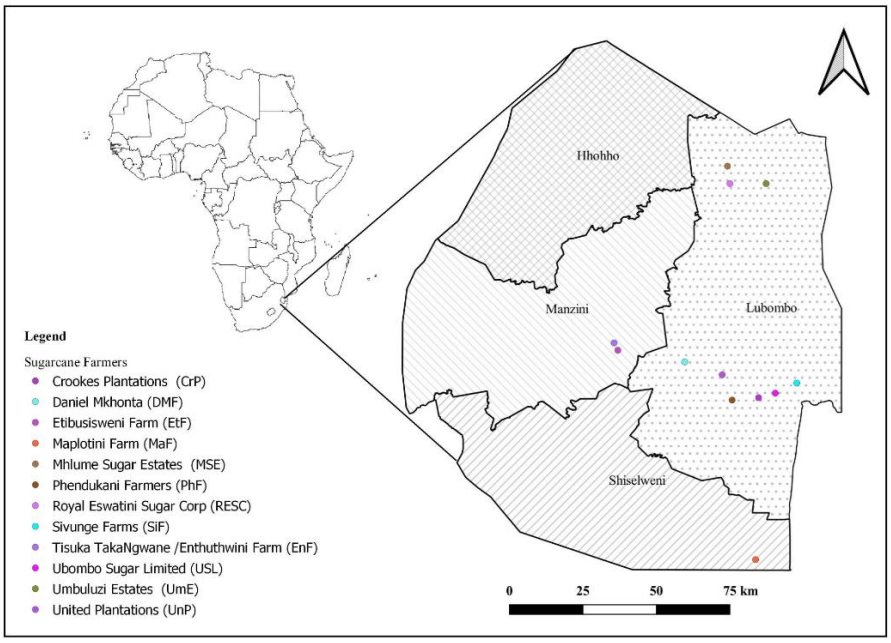

Figure 1: Map of Eswatini showing the locations where *E. saccharina* specimens were collected from sugarcane growers. A smaller inset map indicates Eswatini's location within Africa.

Results

Genetic differentiation

Our analyses used the 603 base pairs region of the cytochrome oxidase sub-unit I gene for which we obtained sequences for all individuals. Levels of sequence divergence among the sampled specimens ranged from 0.0–2.86% (Table 2). Sequence divergences between *E. saccharina* specimens and the out- group taxa ranged from 6.83 to 7.28% (Table 2). There was no evidence of isolation by distance associated genetic differentiation observed. Specimens collected from sugarcane in Lubombo region (United Plantation, Mhlume Sugar Estates and Ubombo Sugar Limited) were identical to specimens collected from sugarcane in the Manzini (Etibusisweni Farm and Enthuthwini Farm) and Shiselweni (Maplotini farm) (Table 2). In contrast, specimens collected from Maplotini farm in Shiselweni region has shown a sequence divergence of 2.68% and were grouped in different clades (Table 2; Fig. 2 and 3).

Table 2. Uncorrected pairwise distances among sequences from sugarcane grower in Eswatini

	Clade I	**Clade II**
Clade I	0.00-0.16	
Clade II	2.52-2.86	0.00
Galleria mellonella	6.83 – 7.05	7.28

Haplotypes

Eleven different haplotypes were identified of which five were unique (i.e. represented by single individuals). Six of the haplotypes (Haplotype 6-11) were recorded only in one grower's farm (Fig. 2). The most common haplotype (Haplotype 1) consisted 34 specimens from nine of the eleven growers visited. Ten of the haplotypes were clustered in one clade with a sequence divergence ranging from 0.08% to 0.16% (Table 2). Haplotype 4 included sequences from four growers and was distant from all other haplotypes and formed its own clade (Fig. 2). There was no clear association between geographic distance and genetic divergence between the 69 individuals of *E. saccharina* in this study. The highest genetic divergence (2.86%) was recorded between specimen 26_UnP (Haplotype 6) and 19_UnP (Haplotype 4), both from United Plantations in Lubombo region, which are geographically very closer to each other. Contrarily, a specimen from in United Plantations (6_UnP) in Lubombo region shared the same haplotype with a specimen from Manzini region (28_EtF) and specimens from Shiselweni region (35-MaF) (Table 1).

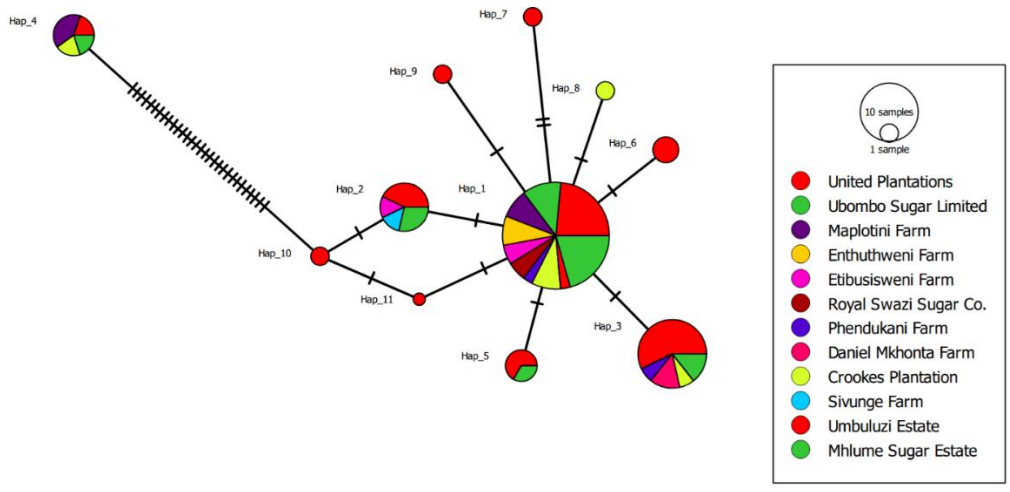

Figure 2. The Median-joining haplotype network of *E. saccharina* genotypes based on the cytochrome oxidase sub-unit I gene. Haplotype composition is shown in pie charts.

The size of the pie chart indicates the number of specimens studied (see Table 1 and legend). The colored sectors represent sources of the specimens (see legend). Haplotypes that differed from each other by a single nucleotide mutation are connected by lines. Each small transverse black line represents a mutation.

Phylogenetic analysis

A Neighbor-joining tree constructed using *Galleria mellonella* as an outgroup grouped the *E. saccharina* sequences into two clades with 89% bootstrap support (Fig. 3). Clade I is the largest group comprising 91% of the haplotypes. This clade has a mixed distribution of haplotypes from nine grower fields distributed in the three estates (Fig. 2, Fig. 3). The haplotypes in the clade were separated by one to two mutational steps from each other. The clade shows within group sequence divergence of up to 0.16% (Table 2). The second Clade II was represented by a single haplotype (Fig. 3). This clade has a sequence divergence of up to 2.86% from the haplotypes in clade I (Fig. 3).

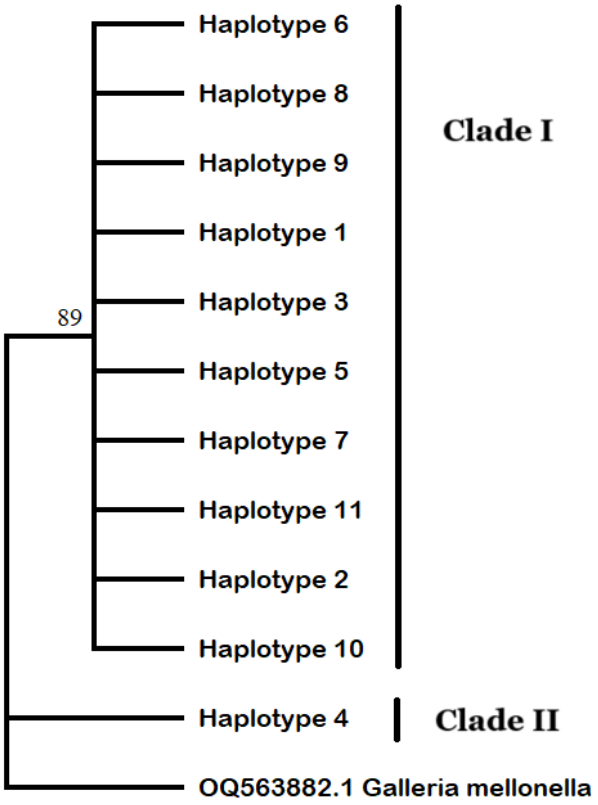

Figure 3. The evolutionary history was inferred using the Neighbor-Joining tree (Saitou and Nei 1987) showing the evolutionary relationships of the eleven haplotypes. The tree is rooted with *Galleria mellonella*. The Number above internode is a bootstrap support value.

Collection localities are indicated on a map of Eswatini (Fig. 1). Haplotype names are indicated in Fig. 2 and Table1.

Discussion

Results of the phylogenetic analyses confirm the separation of *E. saccharina* into two major groups. These findings provide the first molecular evidence of genetic structuring within the Eswatini population of this pest, revealing the existence of two distinct genetic groups despite the relatively small geographic area of the country. The dominant haplotype (Haplotype 1) and the cluster of closely related haplotypes (Haplotypes 1-3; 5-11) that formed clade I were similar to those reported from different countries in southern Africa (Assefa, 2006; Assefa *et al.,* 2010). This population shows a genetic sub-structuring with the nine smaller haplotypes diverging from the dominant haplotype by one to two mutational steps (Fig. 2). The relatively high genetic diversity within this clade could be an indication that this is an old population with a long history in the area. Generally, older, larger populations that have experienced a long history without major bottlenecks or founder effects tend to have greater haplotype diversity. Results showed that *E. saccharina* population in clade I can be considered as an indigenous gene pool within the southern African population of the pest (Assefa ,2006; Assefa *et al.*, 2006; Assefa *et al.*, 2010).

In contrast to the specimens in clade I, specimens included in clade II were genetically distant from clade I but identical to each other, indicating that this is likely a new invasive population. Invasive populations often experience a loss of genetic diversity relative to the source population, due to a small number of founder individuals (Hagan *et al.*, 2024). A small founding population may not reflect the allelic composition of its source population because founding individuals fail to transfer all source alleles to the new population and/or because founder allele frequencies differ strongly from those of the source population (Dlugosch & Parker, 2008). Alternatively, our result likely reflects limited sampling, with this population of *E. saccharina* probably occurring at low frequencies in sugarcane but not in the wild host plants in the natural habitats. As a polyphagous pest, *E. saccharina* feeds on a wide range of grasses and sedges (Assefa, 2006). It is plausible that the specimens from Clade II are adapted to a different host plant that has geographically isolated them from the main sugarcane-feeding population. The association of this population to sugarcane might have occurred recently that there likely has been insufficient

time for genetic differentiation among populations in the introduced host. Several others also showed that recent founder events produced reduction in genetic diversity in the introduced range (Hufbauer *et al.*, 2004; Assefa *et al.*, 2015; Matthee, 2020). Such host-driven specialization has been observed in other insect species, leading to cryptic species formation (Scheffer, 2000; Sezonlin *et al.*, 2006). Further research involving sampling *E. saccharina* from a variety of different host plants would be required to test this hypothesis.

Our results have significant implications for pest management in Eswatini and across the continent. The discovery of two distinct genetic groups highlights the potential for different biotypes of *E. saccharina* to coexist within a small area. This could explain the observed differences in pest behavior, such as varied feeding behavior and responses to natural enemies (Conlong, 2001). The existence of a distinct genetic group (Clade II) warrants caution in the implementation of biological control programs, as a natural enemy effective against the widespread Clade I population might be ineffective against the unique biotype in Clade II. The genetic data generated in this study provide a valuable baseline for future research aimed at understanding the ecological and behavioral differences between these two groups, which is a critical step for developing targeted and effective control strategies.

Conclusion

The phylogenetic analysis of *Eldana saccharina* populations in Eswatini reveals two distinct genetic groups, providing the first DNA-based evidence of genetic structuring for this pest in the country[1]. The study, which used mitochondrial cytochrome *c* oxidase subunit I (COI) gene sequencing on 69 specimens, found significant genetic differentiation between the two groups. The observed genetic differences are likely due to the pest's specialization on different host plants and historical biogeographic events. The study suggests that the Clade II population might be adapted to a different host plant, which has geographically isolated it from the main sugarcane-feeding populationThe findings have significant implications for pest management. The existence of two distinct genetic groups suggests that a biological control program effective against the widespread Clade I population might be ineffective against the unique biotype in Clade II. This highlights the need for further research to understand the ecological and behavioral differences between the two groups to develop targeted control strategies.

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