# GENETIC DIFFERENTIATION IN *ELDANA SACCHARINA* (LEPIDOPTERA: PYRALIDAE): EVIDENCE FROM THE MITOCHONDRIAL CYTOCHROME OXIDASE I AND II GENES

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**Abstract**

The sugarcane stemborer *Eldana saccharina* is an indigenous African moth found throughout much of subsaharan Africa. Previous research has revealed ecological differences among geographical populations, raising the possibility that *E. saccharina* may consist of different biotypes. As a first step towards evaluating this hypothesis, *E. saccharina* populations from across Africa were surveyed for mitochondrial DNA variation. The complete coding regions of the mitochondrial cytochrome *c* oxidase I (COI) and COII genes were sequenced for three individuals from Benin, Uganda and South Africa, and a fragment of COI was sequenced for additional specimens representing a wider geographical range. The levels of sequence divergence found in comparisons between the northern *E. saccharina* populations of Benin, Uganda and Cameroon and those of southern Africa were at least as high as those between recognized biotypes in other species. This suggests potentially limited gene flow among *E. saccharina* populations that merits further investigation.

*Keywords:* molecular systematics, phylogeography, biotypes, cereal stemborer

## Introduction

*Eldana saccharina* Walker (1865) is an indigenous African pyralid moth that is widely distributed throughout much of the continent. Its natural host plants include a range of sedges and grasses. Not surprisingly then, it attacks a variety of graminaceous crops and in southern Africa it has become a serious and widespread pest of sugarcane.

As detailed by Conlong (2001), investigations into *E. saccharina* have led to the following conclusions: not only does *E. saccharina* attack a wide range of host plants, it also favours different plant families in different parts of Africa and the larvae have been found to show differing boring behaviours and to attack different parts of the crop in different parts of Africa. In South Africa it infests sugarcane and attacks the lower third of the stalk. This has also been the case when it has been found in maize, where it is also known to attack old cobs. In West Africa it shows a preference for maize. Surveys conducted in Kenyan sugarcane around Lake Victoria found larvae tend to attack the middle to top third of the stalk, and surveys in Uganda showed a similar pattern (Overholt and Maes, 2000; Conlong, 2001). In addition, natural enemy surveys show that none of the natural enemies of *E. saccharina* collected in West Africa are found in southern Africa (Conlong, 2001). These findings suggest that *E. saccharina* may in fact consist of different biotypes.

Biotypes are distinct lineages of an organism that differ in some aspect of their biology. Thus if biotypes are present, it is not necessarily valid to assume that biological information gathered for

one type is true of another. This has obvious ramifications for the design and implementation of pest control strategies, so it is crucial to know whether biotypes exist. The biological differences defining biotypes are governed by genetic rather than environmental factors, which implies that there must be some limitation to gene flow between biotypes. Biotypes are therefore most reliably diagnosed by genetic differences. DNA sequence analysis has proved to be a useful tool in this regard. Either mitochondrial genes or the internal transcribed spacer (ITS) genes of the nuclear ribosomal DNA repeat are most often used because of their ease of amplification and high rate of evolution.

Mitochondrial DNA fits to a remarkably high degree the description of an ideal molecular marker proposed by Avise *et al.* (1987). It is relatively easy to isolate, even from dried insect specimens, because it exists in multiple copies per cell and the double membranes surrounding mitochondria probably afford a degree of extra protection from degradation. It shows maternal inheritance, which is a particularly important factor in intraspecific studies, as mutations are not obscured by recombination (Brower, 1994; Sperling, 1993). Finally it evolves at a rate 1-10 times faster than the typical single copy nuclear gene (Brower, 1994; Avise *et al.,* 1987). This rapid rate of sequence divergence, especially in the silent sites of mitochondrial protein coding genes, allows recently diverged lines to be distinguished from each other (Harrison, 1989; Simon *et al.,* 1994).

DNA sequences of the mitochondrial cytochrome *c* oxidase genes COI and COII have been used in numerous studies of insect biotypes. For example, Sperling *et al.* (1996) used sequences from the COI-COII gene region to differentiate among four pheromotypes of the dingy cutworm (*Feltia jaculifera*, Noctuidae), Sperling *et al*. (1999) found two distinct lineages of the hemlock looper (*Lambdina fiscellaria*, Geometridae), while Shufran *et al.* (2000) found nine biotypes of greenbug (*Schizaphis graminum*, Aphididae) belonging to three separate host-adapted clades. In this study we present preliminary DNA sequence data from the COI-COII gene region to investigate the possibility that *E. saccharina* consists of different biotypes.

## Materials and Methods

*Material examined*

A summary of material processed is provided in Table 1. For three specimens, Benin DNA extraction no.1, Uganda DNA extraction no.5 and South Africa DNA extraction no.6, the entire 2300bp region was sequenced. For the remaining specimens an approximately 500bp fragment was sequenced. Voucher specimens, including the wings, abdomen and head of each specimen are deposited in the School of Molecular and Cellular Biosciences at the University of Natal, Pietermaritzburg.

*Laboratory techniques*

Total genomic DNA was extracted from the thorax by means of the Qiagen DNeasy™ Tissue Kit. PCR primers were based on Sperling *et al.* (1996). Polymerase chain reaction (PCR) amplifications were performed on a Perkin Elmer GeneAmp PCR System 2400 under the following conditions: 94ºC for 3 minutes, 30 cycles of (94ºC for 30 sec, 55ºC for 30 sec, 72ºC for 30-90 sec), 72ºC for 7 min, 4ºC hold. The reaction volume was 50µl, and the reaction mixture contained: 1X PCR buffer, 2.5mM $MgCl_2$, 200µM of each dNTP, 20 pmol of each PCR primer, 1.25 units of GibcoBRL *Taq* DNA polymerase, and approximately 250ng of genomic DNA/RNA mix. The error rate of *Taq* DNA polymerase is approximately $10^{-5}$ per nucleotide per pass of the polymerase (Cline et al., 1996). Over the course of PCR, in which the polymerase makes an average of 30 passes, the cumulative error rate is roughly $10^{-3}$ per nucleotide. This is insufficient to generate a diverse set of variant sequences, especially over a region shorter than 1,000 nucleotides. Nonetheless, for most PCR fragments both strands were sequenced in order to minimize base misincorporation errors.

**Table 1. Collection data for *Eldana saccharina* specimens used in this study.**

| Collection locality | Collection date | DNA Extraction no. | Sequence direction |
|---|---|---|---|
| Benin | November, 2000 | 1 | Both directions |
| Benin | February, 2000 | 2 | Both directions |
| Benin | November, 2000 | 10 | Single direction |
| Benin | November, 2000 | 11 | Single direction |
| Benin | November, 2000 | 13 | Both directions |
| Cameroon | March, 1999 | 8 | Both directions |
| Cameroon | March, 1999 | 20 | Single direction |
| Uganda | February, 1999 | 5 | Both directions |
| Uganda | February 1999 | 18 | Both directions |
| South Africa[a] | April, 2001 | 4 | Single direction |
| South Africa[a] | April, 2001 | 6 | Both directions |
| South Africa[a] | 2000 | 7 | Single direction |
| South Africa[a] | April, 2001 | 9 | Single direction |
| South Africa[a] | April, 2001 | 22 | Both directions |
| South Africa[a] | April, 2001 | 23 | Both directions |

[a] All South African samples were from the SASEX laboratory colony, whereas other specimens were field collected.

Many *E. saccharina* specimens were stored dry after collection, and as a result only highly degraded DNA could be extracted from them. Therefore the full COI-COII region was amplified in seven short, overlapping PCR fragments. Cycle sequencing was performed using an ABI BigDye™ Terminator v3.0 Cycle Sequencing Kit following the manufacturer's recommended conditions, and sequences were visualized on an ABI 3100 Genetic Analyzer.

*Data analysis*

DNA sequence chromatograms were edited using the Staden package (Staden, 1996). Sequences were automatically aligned using ClustalX (Thompson et al., 1997) and then manually corrected using Se-Al v2.0a7 (Rambault, 2001). The remaining analyses were performed using PAUP*4.0b10 (Swofford, 1998). For the three full length (approximately 2300bp) COI-COII sequences an uncorrected ("p") distance matrix was first obtained. Uncorrected ("p") distance matrices also were calculated for portions of the sequence alignment corresponding to the separate PCR fragments in order to determine which fragment contained the highest sequence variability. This PCR fragment was then sequenced from 11 additional specimens. Phylogenetic analysis was performed under the parsimony criterion. The extent of support for internal nodes was estimated using a 1000-iteration bootstrap analysis.

## Results

*DNA sequence variation*

Three sequences of approximately 2300 bp each were obtained from specimens of *E. saccharina* from Benin, Uganda and South Africa. The Benin and Ugandan specimens differed by only 2 bp, representing a 0.09% sequence divergence. The South African sample differed from the Benin and Ugandan specimens by 18 and 20 bp respectively, representing 0.8% and 0.9% sequence divergences. Most amplified fragments were 300-600bp, with two being approximately 850bp. The most variable of the seven PCR fragments (i.e., the fragment obtained using primers Ron 5 and K525, yielding an approximately 500bp fragment) was selected for analysis of additional specimens.

Fourteen specimens (including the three full length sequences, above) were compared over a short region of approximately 500bp in the COI region. Table 2 shows the uncorrected percentage divergences among these sequences. Sequence SA 6 was identical to SA 9, and the following five sequences also were identical: Benin 1, Uganda 5, Uganda 18, Cameroon 20 and Cameroon 8. Table 3 shows the minimum, maximum and mean percentage divergence values for comparisons within and among geographical areas.

**Table 2. Uncorrected percentage DNA sequence divergence for short fragment, all taxa.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1 SA 6** | - | | | | | | | | | | | | |
| **2 SA 4** | 0.21 | - | | | | | | | | | | | |
| **3 SA 7** | 0.26 | 0.53 | - | | | | | | | | | | |
| **4 SA 9** | 0 | 0.3 | 0.29 | - | | | | | | | | | |
| **5 SA 22** | 0.98 | 0.97 | 1.93 | 1.7 | - | | | | | | | | |
| **6 SA 23** | 0.27 | 0.27 | 0.41 | 0 | 1.29 | - | | | | | | | |
| **7 Benin 1** | 2.07 | 1.86 | 2.3 | 2.24 | 2.62 | 2.16 | - | | | | | | |
| **8 Benin 10** | 2.57 | 2.35 | 2.83 | 2.83 | 3.16 | 2.58 | 0.42 | - | | | | | |
| **9 Benin 11** | 2.42 | 2.21 | 2.78 | 2.71 | 2.61 | 2.15 | 0.23 | 0.7 | - | | | | |
| **10 Benin 13** | 1.86 | 2.07 | 2.04 | 1.95 | 2.63 | 2.17 | 0.21 | 0.65 | 0.44 | - | | | |
| **11 Uganda 5** | 2.07 | 1.86 | 2.3 | 2.24 | 2.62 | 2.16 | 0 | 0.42 | 0.23 | 0.21 | - | | |
| **12 Uganda 18** | 2.08 | 1.87 | 2.3 | 2.23 | 2.64 | 2.18 | 0 | 0.43 | 0.23 | 0.21 | 0 | - | |
| **13 Cameroon 20** | 2.11 | 1.89 | 2.31 | 2.27 | 2.73 | 2.22 | 0 | 0.43 | 0.23 | 0.22 | 0 | 0 | - |
| **14 Cameroon 8** | 2.08 | 1.87 | 2.31 | 2.25 | 2.64 | 2.18 | 0 | 0.43 | 0.23 | 0.21 | 0 | 0 | 0 |

**Table 3. Percentage sequence divergence, summarized by group.**

| Comparison | Min. | Max. | Mean |
|---|---|---|---|
| Within SA | 0 | 1.93 | 0.63 |
| Within Benin | 0.21 | 0.7 | 0.44 |
| Within Cameroon | 0 | 0 | 0 |
| Within Uganda | 0 | 0 | 0 |
| Uganda vs. Cameroon | 0 | 0 | 0 |
| Benin vs. Uganda/Cameroon | 0 | 0.43 | 0.22 |
| SA vs. Uganda/Cameroon | 1.86 | 2.73 | 2.23 |
| SA vs. Benin | 1.86 | 3.16 | 2.38 |
| SA vs. all others | 1.86 | 3.16 | 2.04 |

*Maximum parsimony analysis*

A branch and bound search performed under the parsimony criterion produced 144 trees, with a length of 19 steps, consistency index of 0.95 and retention index of 0.97. The strict consensus tree had only a single branch resolved, that separating the South African specimens from the rest. A bootstrap analysis recovered this branch in 100% of bootstrap replicates. One of the 144 shortest trees is shown in Figure 1 to illustrate the relative length of this branch. Figure 1 was rooted using the midpoint method.

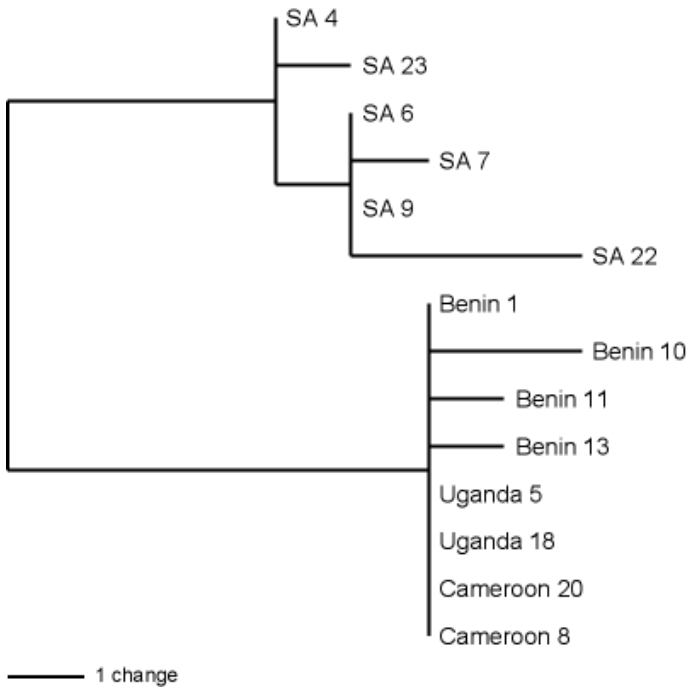


**Figure 1. One of 144 most parsimonious trees, rooted by the midpoint method. Tree length = 19 steps, CI = 0.95, RI = 0.97.**

## Discussion

*Are there different biotypes of* Eldana saccharina?

The most important question is whether the levels of genetic variability among the populations of West, Central, and South Africa are high enough to continue to entertain the possibility of biotypes in *E. saccharina*. The answer to this question must be an unequivocal "Yes." Sperling and Hickey (1994) used COI-COII sequences to investigate the relatedness of the members of the spruce budworm species complex (*Choristoneura* spp.). They claim that a 2.7-2.9% sequence divergence is sufficient to clearly distinguish the oldest species group, while a less than 1% divergence distinguishes only between haplotypes. Analysis of other COI-COII sequences deposited in GenBank shows that even smaller divergences are sometimes found between different species of Lepidoptera. Table 4 shows divergence levels for COI-COII sequence comparisons among selected pairs of closely related lepidopteran species. Clearly, the divergences described in this study fall within that range. While we do not propose that the West African *E. saccharina* populations form a separate species, there is strong evidence for substantial genetic differentiation of those populations. This differentiation is of a sufficiently high level to suggest the existence of biotypes.

**Table 4. Comparison of COI-COII divergence levels among closely related species of Lepidoptera.**

| Species 1 | Species 2 | GenBank Accession No. | Sequence divergence |
|---|---|---|---|
| *Ostrinia nubilalis* | *Ostrinia furnacalis* | AF170853, AF467260 | 1,09% |
| *Archips goyerana* | *Archips argyrospila* | AF309509, AF308931 | 2,29% |
| *Papilio polyxenes* | *Papilio zelicaon* | AF044010, AF044008 | 2,62% |
| *Papilio hospiton* | *Papilio machaon* | AF044009, AF044007 | 3,32% |
| *Papilio zelicaon* | *Papilio hospiton* | AF044008, AF044009 | 3,37% |

It is notable that there is 5-10 times more genetic variability among the six South African samples of *E. saccharina* than among the four Benin samples, or all eight non-South African samples. This is despite the fact that all South African specimens used in this study were obtained from the SASEX laboratory colony. Although this colony is supplemented regularly with field-collected specimens from coastal KwaZulu-Natal, it seems unlikely to represent fully the genetic variability of the southern African region *E. saccharina* populations. The extent to which the SASEX laboratory colony represents field populations of *E. saccharina* is an important question requiring further research. Initial estimates suggest that South African populations exhibit greater genetic variability than West and Central African populations, although current sample sizes are still far too small to draw firm conclusions on this issue.

The maximum parsimony analysis clearly illustrates that there is a significant difference between northern populations (Benin, Uganda and Cameroon) and southern populations (South Africa). However, evidence from ecological studies suggests that the dividing line between these groups is likely to be the Great Rift Valley (Conlong, 2001) which may form a physical barrier to gene flow. If this is indeed the case, then Kenyan populations of *E. saccharina* from the Rift Valley and the coast will be genetically more similar to the Southern African populations than to the Ugandan populations. Populations from the Lake Victoria region of Kenya would, in contrast, be closer to the Ugandan rather than the South African populations. Further research into this question is needed.

*Selection of a suitable marker for analysis*

Relatively few gene regions have been used for phylogenetic studies of insects (Caterino *et al.*, 2001). There is no best molecular character to use in all studies and some genes will suit certain studies better than others (Brower and DeSalle, 1994). The best gene to use largely depends on the level of relatedness being studied and the gene's rate of evolution (Parker *et al.*, 1998). As already discussed, the COI and COII region is often used for this type of phylogenetic study and shows an appropriate evolutionary rate; therefore it was selected for this study. Indeed, the data set very strongly separated one suspected biotype, the South African specimens, from the other, the West and Central African specimens, and shows great promise for further resolving relationships among populations of *E. saccharina*.

*Implications for biological control of* E. saccharina

Because larvae live within the stalk and are protected by it, insecticides are unlikely to be effective against *E. saccharina*. Research has therefore centered on plant resistance, habitat management and finding biological control agents. Of the 23 different indigenous African parasitoids thought to be suitable for biological control of *E. saccharina*, none are found in both West and southern Africa (Conlong, 2001). Although preliminary, these results suggest that it may not be valid simply to apply results of biocontrol research in South Africa to control of *E. saccharina* in West Africa, or vice versa. Further understanding of the biology and behaviour of both *E. saccharina* biotypes and their parasitoids could lead to greater success in biological control.

## Conclusions

The genetic data gathered to date, while not conclusive, provide strong support for the existence of different biotypes within *E. saccharina*. Further research is needed to establish the geographical limits of the suspected biotypes and to assess more thoroughly genetic variability throughout the distribution of *E. saccharina*. Kenyan populations should be sampled in order to better assess the hypothesis that the Great Rift Valley forms a physical barrier to the movement of *E. saccharina*. In addition, field collected specimens must be sampled from throughout Southern Africa. Further data should be gathered from the COI-COII gene region, although more sensitive markers such as ITS sequences or Randomly Amplified Polymorphic DNA (RAPD) markers could provide more resolution within each biotype, if needed.

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## REFERENCES

Avise, JC, Arnold, J, Ball, RM, Bermingham, E, Lamb, T, Neigel, JE, Reeb, CA and Saunders, NC (1987). Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. *Ann Rev Ecol Syst* 18: 489-522.

Brower, AVZ (1994). Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proc Natl Acad Sci USA* 91: 6491-6495.

Brower, AVZ and DeSalle, R (1994). Practical and theoretical considerations for choice of a DNA sequence region in insect molecular systematics, with a short review of published studies using nuclear gene regions. *Ann Entomol Soc Am* 87: 702-715.

Caterino, MS, Cho, S and Sperling, FAH (2001). The current state of insect molecular systematics: A thriving tower of Babel. *Ann Rev Entomol* 45: 1-54.

Cline, J, Braman, JC and Hogrefe, HH (1996). PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases. *Nucl Acids Res* 24: 3546-3551.

Conlong, DE (2001). Biological control of indigenous African stemborers: What do we know? *Insect Sci Applic* 21: 1-8.

Harrison, RG (1989). Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. *Trends Ecol Evol* 4: 6-11.

Overholt, WA and Maes, KVN (2000). *Eldana saccharina* Walker. In: *Field Guide to the Maize and Sorghum Stemborer Larvae of Eastern and Southern Africa*. ICIPE Science Press, Kenya.

Parker, GP, Snow, AA, Schug, MD, Booton, GC and Fuerst, PA (1998). What molecules can tell us about populations: Choosing and using a molecular marker. *Ecology* 79: 361-382.

Rambault, A (2001). Se-Al Sequence Alignment Editor. http://evolve.zoo.ox.ac.uk/Software/Se-Al/main.html

Shufran, KA, Burd, JD, Anstead, JA and Lushai, G (2000). Mitochondrial DNA sequence divergence among greenbug (Homoptera: Aphididae) biotypes: evidence for host adapted races. *Insect Mol Biol* 9: 179-184.

Simon, C, Frati, F, Beckenbach, A, Crespi, B, Liu, H and Flook, P (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann entomol soc Am* 87: 651-701.

Sperling, FAH (1993). Mitochondrial DNA phylogeny of the *Papilio machaon* species group (Lepidoptera: Papilionidae). *Mem ent Soc Can* 165: 233-242.

Sperling, FAH and Hickey, DA (1994). Mitochondrial DNA sequence variation in the spruce budworm species complex (Choristoneura: Lepidoptera). *Mol Biol Evol* 11: 656-665.

Sperling, F, Byers, R and Hickey, D (1996). Mitochondrial DNA sequence variation among pheromotypes of the dingy cutworm, *Feltia jaculifera* (Gn) (Lepidoptera: Noctuidae). *Can J Zool* 74: 2109-2117.

Sperling, FAH, Raske, AG and Otvos, IS (1999). Mitochondrial DNA sequence variation among populations and host races of *Lambdina fiscellaria* (Gn) (Lepidopetra: Geometridae). *Insect Mol Biol* 8: 97-106.

Staden, R (1996) The Staden Sequence Analysis Package. *Mol Biotech* 5: 233-241.

Swofford, DL (1998). PAUP* Phylogenetic analysis using parsimony *(And other methods). Sinauer Associates, Sunderland, Massachusetts.

Thompson, JD, Gibson, TJ, Plewniak, F, Jeanmougin, F and Higgins, DG (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nuc Acids Res* 24: 4876-4882.