

# DIFFERENCES IN MITOCHONDRIAL DNA AND FERTILITY OF CROSSES BETWEEN POPULATIONS OF *ELDANA SACCHARINA* (LEPIDOPTERA: PYRALIDAE) FROM KENYA AND SOUTH AFRICA: POSSIBLE EVIDENCE FOR CRYPTIC SPECIES?

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## Abstract

Genetic differentiation in two populations of *Eldana saccharina* Walker, a Rift Valley population from *Cyperus papyrus* L. bordering Lake Naivasha in Kenya and a South African population from sugarcane, sedges (*Cyperus* spp.) and laboratory colonies at SASRI was examined. To explore the phylogeographical relationships of the populations, 561 bp of mitochondrial DNA (mtDNA) was sequenced from 13 individuals. All individuals from Kenya shared a common haplotype, and the South African sequences were clustered under a second haplotype. These populations have a sequence divergence equivalent to sequence divergences found in sister species. Could this be the first evidence of a cryptic species within *E. saccharina*? To examine this hypothesis, inter-population crosses were made to produce an F<sub>1</sub> generation, and these were backcrossed with the South African parent population. Fertility of eggs produced by the F<sub>1</sub>/parent population cross was significantly reduced when compared with fertility of the 'true' SA line, and the F<sub>1</sub>/ F<sub>1</sub> cross. The mating experiments thus suggest that the Kenyan and South African populations are at least partially reproductively isolated. This is consistent with the large DNA distances observed in mtDNA sequences. The infertility of the hybrids followed Haldane's rule, where heterozygotic females are more infertile than the males.

*Keywords:* mitochondrial DNA, *Eldana saccharina*, Rift Valley, stalk borers, genetic relationships

## Introduction

Understanding the process of speciation amounts to understanding the process of reproductive isolation (Christianson *et al.*, 2005). Factors inhibiting mating, fertilisation, fitness and fertility of organisms create barriers to gene flow. The isolated populations accumulate genetic differences with time that contribute to speciation. The invention of PCR technology and the introduction of mitochondrial and microsatellite DNA markers has made studies on genetic differentiations remarkably easy, fast and successful (Hillis *et al.*, 1996).

Mitochondrial DNA (mtDNA) has proven powerful for genealogical and evolutionary studies of animal populations, and it is the most commonly employed marker for determining genetic relationships among populations (Sperling *et al.*, 1999; Scheffer, 2000; Scheffer and Lewis, 2001; Segraves and Pellmyr, 2001; King *et al.*, 2002; Simmons and Scheffer, 2004). Due to maternal inheritance and a relatively faster rate of evolution, mtDNAs have been used to

provide insights into population genetic structure, gene flow, biogeography and intraspecific relationships (Danforth *et al*, 1998; Moritz *et al*, 1987; Sperling *et al*, 1999; Simmons and Scheffer, 2004). Such data are also capable of revealing cryptic lineages representing distinct species within geographically widespread and apparently morphologically homogeneous organisms (Scheffer, 2000). However, results of mtDNA analysis will be more meaningful when combined with other data. Information on morphology, ecology, hybridisation and differences in nuclear DNA are used, together with mtDNA markers, in studying intraspecific and interspecific genetic relationships between organisms (Simmons and Scheffer, 2004; Jenkins *et al*, 2001; Parkinson *et al*, 2000; Sperling, 1993). This study examines genetic differentiation in two geographically isolated populations of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) and investigates the effect of the genetic differentiation on the fertility of inter-population crosses.

## Materials and Methods

### *Insect specimens*

*Eldana saccharina* specimens used in the experiments were obtained from infested *Cyperus papyrus* L. (Cyperaceae) bordering lake Naivasha in the Rift Valley in Kenya, from sugarcane and sedges (*Cyperus* spp.) in different parts of South Africa and from the South African Sugarcane Research Institute (SASRI) insect rearing unit. The founding population for the SASRI colony were collected from various sites in the KwaZulu-Natal Province of South Africa, and the colony is regularly supplemented by field collected specimens from this region. The study was conducted in two phases. In the first phase, DNA sequence analysis of regions of the mitochondrial Cytochrome Oxidase c subunit I gene (COI) was performed on the 13 *E. saccharina* samples collected from the two countries. The second experiment was on the fertility of the hybrids from the two geographically isolated populations. The time of collection, locality and host plants of the specimens examined in the mtDNA study is indicated in Table 1, along with the DNA number of each sequence reported in this study.

**Table 1. Collection data for *Eldana saccharina* Walker (Lepidoptera: Pyralidae) specimens used in this study.**

DNA No.	Country	Location	Latitude, Longitude	Year	Host
78	Kenya	Lake Naivasha	0°45'S and 36°25'E	2002	<i>Cyperus papyrus</i>
85	Kenya	Lake Naivasha	0°45'S and 36°25'E	2002	<i>Cyperus papyrus</i>
86	Kenya	Lake Naivasha	0°45'S and 36°25'E	2002	<i>Cyperus papyrus</i>
4	South Africa	SASRI Colony	29°42'S and 31°02'E	2001	N/A
6	South Africa	SASRI Colony	29°42'S and 31°02'E	2001	N/A
7	South Africa	SASRI Colony	29°42'S and 31°02'E	2000	N/A
22	South Africa	SASRI Colony	29°42'S and 31°02'E	2001	N/A
23	South Africa	SASRI Colony	29°42'S and 31°02'E	2001	N/A
79	South Africa	Gingindhlovu West	29°02'S and 31°30'E	1998	Sugarcane
80	South Africa	Richards Bay	28°48'S and 32°06'E	1998	<i>Cyperus papyrus</i>
83	South Africa	Table Mountain, KZN	29°35'S and 30°30'E	2002	Unknown
300	South Africa	Mtunzini Farm	28°57'S and 31°39'E	2002	<i>Cyperus dives</i>
301	South Africa	Mtunzini Farm	28°57'S and 31°39'E	2003	<i>Cyperus dives</i>

N/A = not applicable

All samples from SASRI colony were included in a previous study by King *et al*. (2002).

### *Experiment I: Mitochondrial DNA sequence divergence*

Genomic DNA was extracted from individual thoraxes using the Qiagen DNeasy™ Tissue Kit as recommended for DNA isolation from animal tissue, and the extracted DNA was stored at  $-20^{\circ}\text{C}$  until required for amplification. Polymerase Chain Reaction (PCR) amplification was performed in a 50  $\mu\text{l}$  volume containing 1X PCR buffer, 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  dNTPs, 15 pmol of each PCR primer, 1 unit of SuperTherm Gold *Taq* DNA polymerase and 1  $\mu\text{l}$  of genomic DNA. PCR was performed using a Perkin Elmer GeneAmp PCR System 2400, using the reaction conditions detailed in King *et al.* (2002). 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, cleaned using Ethanol/EDTA precipitation with slight modification of the manufacturer's protocol, and sequences were visualised on an ABI 3100 Genetic Analyzer. Primers used in the study were as mentioned in King *et al.* (2002).

Editing and assembling DNA sequence chromatograms was done using the Staden package (Staden, 1996). Sequences were then aligned using ClustalX (Thompson *et al.*, 1997) and manually corrected using BioEdit sequence alignment editor (Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Haplotype network was constructed using TCS1.2 software at nine connection steps and 95% connection limits. Phylogenetic analysis was performed by Maximum Parsimony (MP) in PAUP\* v4.0b10 (Swofford, 2002) using heuristic search, tree-bisection-reconnection (TBR) swapping, and comprised 100 random addition sequence starting trees. Tree reliability was assessed by bootstrap analysis with 1000 replications.

### *Experiment II: Inter-population mating*

Laboratory colonies were initiated with three moths that emerged from pupae collected from *C. papyrus* bordering lake Naivasha, Kenya. These Kenyan moths were cross-mated with individuals of the opposite sex from the South African laboratory colony. The  $F_1$  hybrid broods were reared simultaneously with control broods of parental stock from the SASRI insect unit through to adults. The  $F_1$  hybrid adults were then backcrossed with adults of the opposite sex from the parental type control broods, and cross-mating among hybrids and  $F_1$  adults of the parental broods were also completed. The types of crossings were:

- $F_1$  Hybrid Male x  $F_1$  Hybrid Female (4 pairs)
- $F_1$  Hybrid Male x Control Female (13 pairs)
- Control Male x  $F_1$  Hybrid Female (11 pairs)
- Control Female x Control Male (9 pairs).

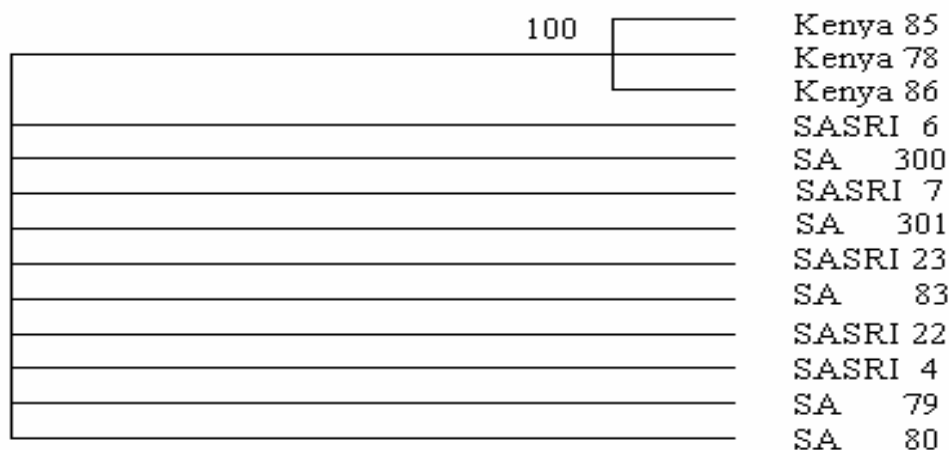
The colonies were maintained under standardised conditions developed for *E. saccharina* rearing (Graham and Conlong, 1988). Larvae were reared individually in 30 ml plastic vials containing artificial diet. The vial was sealed with a perforated lid. The perforation was covered with very fine mesh stainless steel gauze. The vials were checked weekly and the larvae were transferred to new vials to replace the artificial diet until they pupated. Pupae were placed in a 90 mm diameter plastic petri dishes for adult emergence. Emerged adults were immediately paired and pairs of moths were placed in 750 ml plastic jars with very fine mesh stainless steel gauze lid containing 22x28  $\text{cm}^2$  paper towel that was tightly folded to enhance egg laying (Dick, 1945) and the paper towel was replaced every day. Paper towels

with egg masses obtained from cross-matings were heat sealed in a polyethylene plastic bag about 20x26 cm<sup>2</sup> in size to ensure that there was enough air for the eggs until they hatched. The polyethylene bags were then labelled and placed in an incubator at 24±1°C and 75% relative humidity until neonate emergence. Egg masses were monitored daily for hatching for 15 consecutive days (Dick, 1945). A pair was referred to as fertile when at least one larva emerged from eggs laid throughout the lifetime of the female, and referred to as unfertile otherwise. The proportion of fertile pairs for each treatment was obtained by dividing the fertile pairings to the total number of pairings for that particular treatment. These proportions were converted to percentages and referred to as percentage fertility. The significance of the differences in fertility observed between the different types of crosses was analysed using Generalised Linear Model (GLM), with a binomial distribution and a logit link function in Genstat software (Genstat (2005). Genstat, 8th Edition. VSN International Ltd. <http://www.vsn-intl.com/genstat/>).

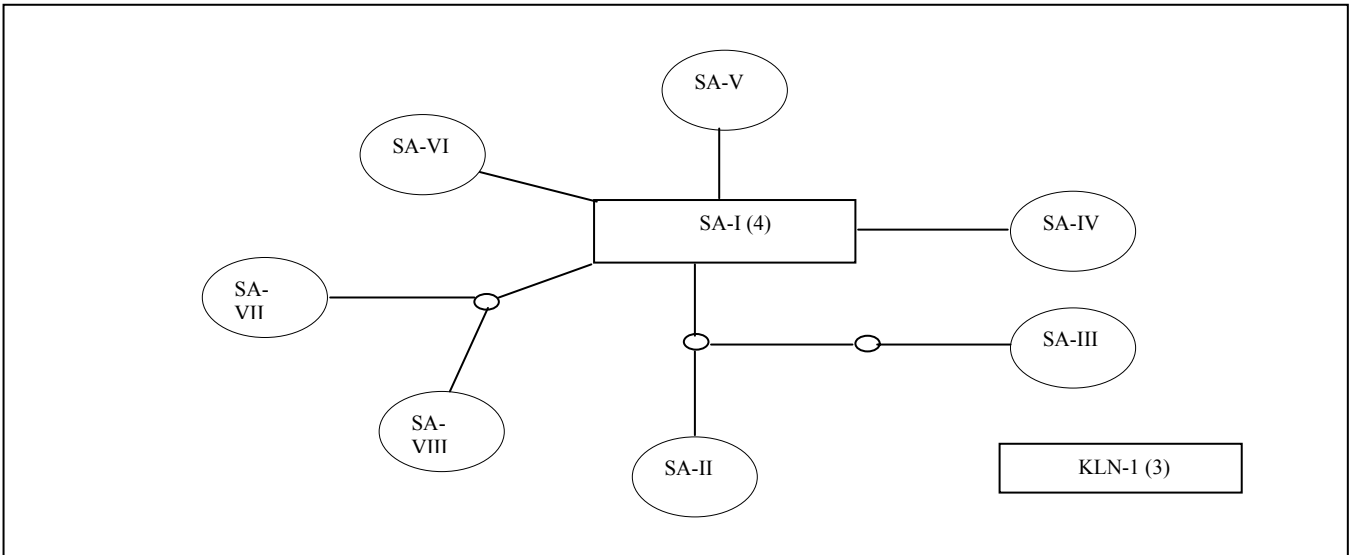
## Results

### *Mitochondrial DNA sequence divergence*

The strict consensus Maximum parsimony tree (length=28; retention index=0.9773) of the 13 individuals analysed is shown in Figure 1. The South African and Kenyan sequences were separated into two groups: All Kenyan sequences fell into one haplotype and the South African sequences were clustered around another haplotype with only one to two mutational differences from each other (Figure 2). The most common South African haplotype was represented in four of the sequenced individuals and the remaining South African sequences were unique. Twenty-seven variable sites were found in the 561 bp fragment for which all specimens had data. Of these, 22 sites were parsimony-informative. Uncorrected pairwise sequence distances ranged from 0 to 4.1% (Table 2).



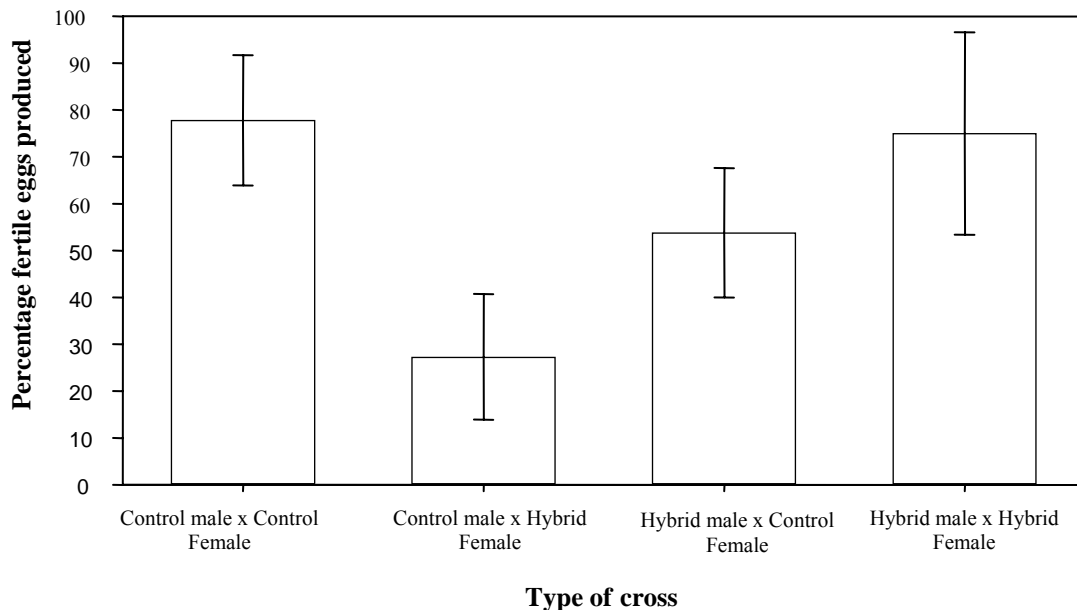
**Figure 1. Strict consensus tree representing mtDNA relationships of the specimens of *E. saccharina* Walker (Lepidoptera: Pyralidae). Number above internodes is value of bootstrap support. Numbers next to the country names are DNA numbers indicted in Table 1.**



**Figure 2: Haplotype network showing the relationship between South African and Kenyan sequences. Numbers in brackets are the numbers of individuals that fall under the haplotype, and circles represent the unique haplotypes that are represented by roman numerals from I to VIII. One node represents one mutational change and unconnected haplotypes are far from each other by more than nine mutational steps. SA=South Africa; KLN=Kenya Lake Naivasha.**

*Inter-population mating*

Results of the backcrossing between the F<sub>1</sub> hybrid (Kenyan x South African) and the parental South African laboratory colony of *E. saccharina* are shown in Figure 3. Despite the genetic distance between the Kenyan and South African populations of *E. saccharina*, the crosses gave viable offspring. However, the fertility rates of the backcrosses were significantly lower (LSD=0.05). Fertility of the cross between control males and F<sub>1</sub> hybrid females was three times lower than the control and between F<sub>1</sub> hybrid crossings.



**Figure 3. Percentage fertile eggs laid following backcrossing hybrid F<sub>1</sub> broods with South African parental brood. The error bars in the bar graph show the standard errors of the treatments.**

Only three of the 11 pairings (27.3%) between control males and F<sub>1</sub> hybrid females produced fertile eggs. Backcrosses between the male F<sub>1</sub> hybrids and control females also showed a reduction in fertility. However, the fertility of these matings was almost double that of the control male and F<sub>1</sub> hybrid female matings. In these backcrossings, seven pairings out of 13 (53.85%) produced fertile eggs. There was no difference observed between hybrid/hybrid and control/control matings (Figure 3).

### Discussion

Results of haplotype networking and phylogenetic analyses confirm the separation of *E. saccharina* into two major groups corresponding to their geographical position (Figures 1 and 2). One group is localised in South Africa (SA) and the other group restricted to Kenya (KLN I). Conlong (2001) suggested that *E. saccharina* might have undergone significant genetic differentiation, as his ecological studies showed that the insect exhibited considerable behavioural variation, displaying differential responses to indigenous control agents and host plants in various parts of Africa. The results presented in this paper support his hypothesis. The uncorrected pairwise divergences measured within the African *E. saccharina* populations in this study suggest the presence of distinct lineages. Variation in COI observed between the Kenyan specimens from Lake Naivasha and the South African specimens (up to 4.1%; Table 2) is at least equivalent to what was previously reported between many other pest species by Sperling *et al.* (1999), Evans *et al.* (2000) and Scheffer and Lewis (2001).

**Table 2. Percentage uncorrected pairwise distances within and between sequences from Kenya and South Africa.**

	Kenyan Group	South African Group
Kenyan Group	<b>0.0</b>	
South African Group	3.7-4.1	<b>0.0-0.7</b>

Within-group pairwise distances are shown in bold on the diagonal and the value below them represents between-group distance.

This differentiation between the two groups suggests that *E. saccharina* may contain a cryptic species. However, it is imprudent to infer that the observed genetic differences reflect interspecific diversity without more, and different, supporting evidence. As reported by Landry *et al.* (1999), percentage mtDNA sequence divergence between closely related sister species of Lepidoptera is highly variable, and is not necessarily a good predictor of whether two unknown populations constitute reproductively isolated species. The cross-mating experiment conducted to confirm mtDNA evidence clearly showed that the genetic differentiation observed has an impact on the fertility of the cross-matings. This infertility in backcrosses could be explained by the existence of pre or post-zygotic reproductive barriers, which would prevent, in free choice situations as in nature, the coupling of moths from different regions. Any matings between these different populations would result in reduced viability of the progeny, and affect the fitness and survival of the offspring (Christianson *et al.*, 2005; Wu and Davis, 1993; Oliver, 1979).

Wide variation in fertility of F<sub>1</sub> hybrid broods backcrossed with the parental brood was observed in this study. Infertility of F<sub>1</sub> hybrid females was much higher than that of F<sub>1</sub> hybrid males crossed with control individuals of the opposite sex. The pattern of reproductive isolation observed in this experiment follows Haldane's rule where the heterozygotic sex in the interspecific hybridisation is affected (Wu and Davis, 1993; Oliver, 1979). Studies

conducted on the mtDNA differences and Haldane's rule in other Lepidoptera, where females are heterozygotic, shows a strong correlation between mtDNA variation and the extent of the Haldane effect, as well as with the presence of major species differences on sex chromosomes (Sperling, 1993). Therefore, the correlation between distinct mtDNA populations and Haldane's rule observed may indicate the presence of a cryptic species within *E. saccharina*.

### Conclusions

Results of the current study have shown the existence of considerable genetic differentiation between populations of *E. saccharina* from Lake Naivasha, Kenya, and from KwaZulu-Natal, South Africa. Fertility of offspring from cross-mating studies with F<sub>1</sub> hybrids from these populations strongly support the genetic differences measured, as the F<sub>1</sub> hybrid/control population crosses were significantly less fertile than the pure control population crosses. However, the reasons for the hybrid infertility observed are not yet clearly identified, and the impact of the genetic differentiation on the longevity and survival of the backcrosses is not known. Therefore, it is important to study factors inhibiting pre-mating and post zygotic isolation, and to evaluate the impact of the genetic differentiation on survival and longevity of the backcrosses before the status of the populations can be clearly defined. In addition, increased sampling and analysis of individuals, at both the geographic and host plant levels, needs to be completed to clearly determine the possible existence of cryptic species.

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