Ecology and population genetic structure of strains of *Teretrius Nigrescens* (Coleoptera: Histeridae), predator of *Prostephanus truncatus* (Coleoptera: Bostrichidae)

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Thesis submitted in fulfilment of the requirements for the award of the degree Doctor of Philosophy in Environmental Sciences at the North West University (Potchefstroom Campus)

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May 2009

DEDICATION

To

Anneke Leeuwenberg,

Friend without borders

Who, sadly, must now take a rest.

ACKNOWLEDGEMENTS

This work benefited from the support of many people to whom I am sincerely grateful. First and foremost, I appreciate the guidance, patience and encouragement by my supervisors Prof. Johnnie van den Berg, Drs. Fritz Schulthess and Daniel Masiga who, together with The Late Dr. Charles Omwega, taught me so much and nurtured my fledgling skills. I appreciate the immense logistical, moral and surrogate supervisory support of the Noctuid Stemborer Biodiversity team and Dr. Bruno Le Rü, who hosted this project in its final one year. I appreciate the statistical input of Dr. Nanqing Jiang (UNEP, China) for modelling work, Dr. Emese Meglécz (Université de Provence, Marseille) in MICROFAMILY analysis and Dr. Bruce Anani for sundry statistical support.

Our collaborators' support and contribution to this work is greatly appreciated. Dr. Josephine Songa, Dr. Francis Nang'ayo and Mr. Paddy Likhayo started us up with colony establishment and management; while Messrs. Robert Mutweti, David Makumi and Patrick Lumumba helped with monitoring LGB fieldwork. The strains of the predator used were donated by Dr. David Bergvinson (CiMMYT, Mexico); Cyprian Atcha (IITA – Benin), Rhodrick Ndawala (NARO – Malawi) and Dr. Richard Hodges (NRI, UK) sent us samples from UK and offered lots of advice. Partnership with Dr. Alfredo Rueda and Ms. Lucia Orantes (Zamorano University, Tegucigalpa) enabled us to prospect for three new strains of *T. nigrescens* from Honduras. Dr. Jeffrey Grubber (Russel Laboratories, Madison) sent us samples of sister group species. Drs. Pierpaolo Vienna and Riaan Stals identified the samples and together with the Histeridologists Network, advised on outgroup species. Thank you all for your kindness.

My heartfelt appreciation to the staff of Biosciences East and Central Africa BecA hub, ILRI: Moses Naira, Delia Wasawo, Julius Osaso, Lucy Muthui, Bernard Waswa, Mercy Kitavi and Dr. Rob Skilton for guidance during my sojourn at ILRI Laboratories. Support from Drs. Pedro Fernandes (Gulbenkian Institute, Lisbon) Paul-André Calatayud and George Ongamo expanded the available information vastly. I also appreciate the input of all my colleagues: Drs. Kipkoech, Muli, Fenning-Okwae and, Obonyo, Migiro, Niassy and all the others made this work manageable. My laboratory colleagues especially Mr. Kabii, Dr. Eric Kouam, David Wainaina, Dr. Paul Mireji, Steve Ger, Eunice Machuka, Pamela Seda, Fathiya Khamis and Vincent Owino.

My profound gratitude to Sarine Adhiambo, Charles Kamonjo and Andrew Wanyonyi, who between, them maintained the insect colonies and helped with the experiments. I am also grateful for technical assistance from Gerphas Okuku, Julius Obonyo, David Wainaina and all my student-interns. Administrative support by Ms. Caroh Akal and Beatrice Gikaria (Biocontrol Project) the Capacity Building Office and Molecular Biology and Biotechnology Unit made our work smooth. I am also grateful to my family: Mother, Paschalia Auma Oduor, sisters Beatrice Achieng' and Anne Akinyi and brothers Clement Argwings and Stephen Israel for urging on these efforts. To the many people who helped in diverse ways, but who I did not mention by name, thank you very much. *Nothing is too small*. The work of your helping hands lives in the submission of this thesis.

Finally I am hugely indebted to the Kirkhouse Trust, UK for a generous sponsorship my PhD Fellowship and for providing research funds; the Swiss Government for funding the prospecting for samples in Honduras; and BecA-Hub, ILRI-Kenya for Research Fellowship for access to ILRI molecular Biology laboratories.

Above all, I thank God, by who’s Grace all was possible.

ABSTRACT

The larger grain borer (LGB) *Prostephanus truncatus* (Horn) is the most important pest of farm stored maize and cassava in Africa. This alien invasive species was introduced into the continent from Mesoamerica in the late 1970s and by 2008 had spread to at least 18 countries. In contrast to indigenous primary storage pests, LGB exists as on-farm and as wild populations, hence, sustainable control must target both environments. Biological control is especially attractive for wild populations to reduce early season grain store infestation, while cultural and chemical methods are useful to protect stored produce directly. Two populations of the predator *Teretrius nigrescens* Lewis were introduced into several African countries as a biocontrol agent. It has shown long-term success and cost effective control in warm-humid areas. Control has however not been successful in cool and hot-dry zones. The aim of this study was to investigate the possible underlying genetic and ecological explanations for these observations and the possibility of joint use of molecular markers and ecological parameters in the development of sustainable control strategies. A 28-month baseline monitoring and recovery activity was done in from 2004 in five regions in Kenya along an east-westerly transect. Monitoring and live sample collection was also done in the original outbreak area in eastern Kenya. There was greater LGB flight activity in western Kenya (high potential maize production area) than the low potential areas. Very few *T. nigrescens* were recovered, solely in the eastern regions. LGB flight activity followed a seasonal pattern mostly related to changes in the relative humidity at 12:00, rainfall and dew point temperature but with a 3 - 4 week lag. A linear predictive model based on these factors predicted 27 % of the observed flight activity. The survival and predation of five strains of *T. nigrescens* were compared at eight temperature levels between 15 °C and 36 °C at low and high humidity. All the strains of *T. nigrescens* exerted a significant reduction of LGB population build-up between 21 °C and 33 °C with generally better performance under humid conditions. There was no evidence

of *T. nigrescens* development at 15 °C. At 18 °C, *T. nigrescens* oviposition and development was observed but the effect on LGB did not differ significantly from the control. The KARI population was the least effective in preventing grain damage at lower temperatures, but performed better than other strains above 30 °C at low humidity conditions. There was no control at 18 °C and 36 °C under both high and low humidity conditions. Since the extent of genetic differentiation in *T. nigrescens* was unclear from prior studies, several molecular marker techniques were progressively used. The RAPD-PCR did not reveal any genetic diversity between geographical populations. A 1000bp region of the mitochondrial mtCOI gene revealed two distinct clades differing consistently at 26 segregating sites. The two clades can be identified by simple PCR-RFLP procedure using single or double sequential restriction with *Eco*R1, *Hinc*II, *Rsa*I and *Dde*I digestion. However, the two lineages co-exist among the mid-altitude Central American populations. The internal transcribed spacer regions ITS1 and ITS2 with some neighbouring coding sequences of the ribosomal DNA were cloned and sequenced. The spacer regions were so variable in length and sequence between *T. nigrescens* and related Histeridae species that direct sequence alignment was not meaningful. Within *T. nigrescens*, there was intragenomic variability of the spacer regions mostly involving insertions and deletions of variable tandem repeat units predominantly within the ITS regions. The short flanking coding (18S, 5.8S and 21S) regions were conserved across populations and six other Histeridae species. There was no significant secondary structure variation of the ITS regions among populations of *T. nigrescens*. Twenty-four novel variable microsatellite markers were developed and tested on the Honduras populations. Alleles per locus ranged between two and twelve with observed heterozygosity between 0.048 and 0.646. Six loci deviated significantly from Hardy Weinberg Equilibrium and possibly had null alleles. The success of microsatellite amplification in outgroup species and variability of markers declined with an increase in the phylogenetic distance between the test species and *T. nigrescens*. Genotyping 432 individuals

from 13 geographic populations revealed a comparatively higher genetic diversity in field populations. Partial isolation by distance and time was observed. Population bottlenecks were not detected, but recent expansion was evident in laboratory populations. Although five dominant genetic clusters were identified by Bayesian methods, meaningful hierarchical population structure was observed at between two and nine population groups ($p < 0.01$; 10,000 iterations). Biological control of the larger grain borer using *T. nigrescens* seems an important aspect of the sustainable integrated control approach of the pest. Ecological adaptations, appropriate release strategies and genetic diversity are all essential considerations in these efforts and could be responsible for the variable success already observed. There is some genetic differentiation between populations of *T. nigrescens* but, further studies would be necessary to ascertain the contribution of such diversity to its predatory performance. The effect of laboratory culturing in aggravating genetic drift should be accommodated to avoid loss of diversity during sampling, quarantine, rearing and release of the predator.

Key words: *Teretrius nigrescens, Prostephanus truncatus*, genetic differentiation, molecular markers, DNA sequence analysis, microsatellites, ecological suitability.

UITTREKSEL

Die groot graanboorder (GGB), *Prostephanus truncatus* (Horn), is die belangrikste plaag van plaasgestoorde mielies en cassava in Afrika. Hierdie eksotiese indringerspesie is per abuis gedurende die laat 1970s vanaf meso-Amerika na die die Afrika-kontinent gebring en het sederdien versprei na ten minste 18 lande. In teenstelling met inheemse plae van gestoorde graan bestaan GGB beide op-plaas en as wilde populasies wat tot gevolg het dat ‘n volhoubare beheerstrategie beide omgewings sal moet teiken. Biologiese beheer is veral geskik om vroeë-seisoen infestasies in graanstore te beperk, terwyl kulturele en chemiese beheermetodes nuttig is om die produk direk te beskerm. Twee populasies van *Teretrius nigrescens* Lewis is na verskillende Afrika-lande ingevoer en het langtermyn sukses en koste-effektiewe beheer van GGB in warm humide areas tot gevolg gehad. Beheer was egter nie effektief in koel en droë areas nie. Die doel van hierdie studie was om moontlike onderliggende genetiese en ekologiese verklarings vir hierdie waarnemings te ondersoek asook om die moontlike geïntegreerde gebruik van molekulêre merkers en ekologiese parameters in die ontwikkeling van volhoubare biologiese-beheerstrategieë te ondersoek. Feromoonvalle is gebruik om populasies van GGB en *T. nigrescens* te monitor oor ’n periode van 28 maande langs ’n Oos – Wes gradient in vyf streke van Kenia. Monitering asook versameling van lewende materiaal is gedoen in die oorspronklike uitbraak-area in die ooste van Kenia. GGB is versamel in alle streke in die land. Daar was groter vlugte in die westelike hoë-potensiaal mielieproduskiegebiede as in lae-potensiaalgebiede. *T. nigrescens* is slegs in die oostelike streke opgespoor en in lae hoeveelhede. GGB vlugaktiwiteit volg ’n seisoenale patroon wat grootliks verband hou met veranderinge in relatiewe humiditeit teen 12:00, reënval en doupunt-temperatuur maar met ‘n 3-4 week sloering in respons tot verandering in hierdie kondisies. ’n Liniêre voorspellingsmodel gebaseer op hierdie faktore het 27 % van die waargenome vlugaktiwiteit voorspel. Die oorlewing en predasie van vyf biotipes van *T.*

nigrescens is vergelyk by agt verskillende temperature tussen 15 °C en 36 °C. Alle biotipes van *T. nigrescens* het ‘n betekenisvolle afname in GGB populasie-opbou veroorsaak tussen 21 °C en 33 °C. Geen ontwikkeling van *T. nigrescens* is by 15 °C waargeneem nie. By 18 °C is *T. nigrescens* eierlegging en ontwikkeling waargeneem maar dit het nie verskil van die kontrole nie. Die KARI-populasie was die minste effektief in die voorkoming van graanskade by laer temperature maar het beter gedoen as die ander biotipes by temperature bo 30 °C en by lae humiditeit. Geen beheer is waargeneem by 18 °C en 36 °C onder beide hoë en lae humiditeit nie. Aangesien die omvang van genetiese variasie in *T. nigrescens* nie duidelik was vanuit vorige getuienis nie, is verskeie molekulêre tegnieke oor tyd gebruik om hierdie aspek te ondersoek. Die RAPD-PCR-tegniek het nie enige genetiese differensiasie of duidelike potensiële reeksgemerkte lokusse aangedui nie. ‘n 1000bp streek van die mitokondriale SO1-geen het twee duidelik onderskeibare klades van die predator aangetoon wat deurgaans op 26 segregerende lokusse verskil het. Twee klades kan identifiseer word deur eenvoudige PCR-RFLP prosedures deur gebruik te maak van enkel of dubbel-opvolgende restriksie met *Eco*R1, *Hiinc*II, *Rsa*I en *Dde*I. Twee lyne word egter gedeel tussen die mid-sentraal Amerikaanse populasies. Die interne getranskribeerde spasiestreke ITS1 en ITS2, tesame met die naburige koderende reekse van die ribosomale DNA is geamplifiseer, gekloon en die volgorde bepaal. Die spasiestreke tussen *T. nigrescens* en aanverwante Histeridae spesies het so varieer in lengte en volgorde dat direkte volgorde-sinkronisasie nie moontlik was nie. Selfs binne *T. nigrescens* is intergenoomvariasie van die spasiestreke, wat meestal veranderlike tandem herhaalde eenhede behels, waargeneem. Die meerderheid van die variasie was binne die ITS1 opvolgreeks. Die “kort kant” sykant koderings-streke (18S, 5.8S and 21S) is bewaar oor populasies en ses ander Histeridae spesies. Daar was geen betekenisvolle sekondêre struktuurvariasie van die ITS-streke tussen populasies van *T. nigrescens* nie. Vier-en-twintig nuwe veranderlike mikrosattelietmerkers is ontwikkel om enige onlangse demografiese gebeurtenisse in *T. nigrescens* populasies te raam. Hierdie

merkers is getoets op ’n veldpopulasie vanaf Honduras. Die aantal allele per lokus het varieer tussen twee en 12 met ’n waargenome heterosigositeit tussen 0.048 and 0.646. Ses loci het betekenisvol afgewyk vanaf die Hardy Weinberg Ekwilibrium en het aangedui dat daar geen amplifiserende allele voorkom nie. Die loci het die veranderlike allele in sewe ander Histeridae spesies geamplifiseer maar die sukses en veranderlikheid het afgeneem met ’n toename in die poligenetiese afstand tussen die toetsspesie en *T. nigrescens*. ’n Groter genetiese diversiteit is by Genotipe 432 onder veldtoesande aangetoon. Gedeeltlike isolasie oor afstand en tyd is waargeneem. Geen bottelnekke is in populasies waargeneem nie maar onlangse uitbreiding is waargeneem in laboratoriumpopulasies. Alhoewel vyf dominante genetiese groepe geidentifiseer is met behulp van Bayesiaanse metodes, is betekenisvolle hierargiese populasiestrukture waargeneem tussen twee en nege populasiegroepe ($p < 0.01$; 10,000 iterasies). Biologiese beheer van GGB met *T. nigrescens* is ‘n belangrike komponent van ‘n volhoubare geïntegreerde beheerstrategie. Ekologiese aanpassings, toepaslike vrylatingsstrategieë en genetiese diversiteit is noodsaaklike oorwegings in hierdie pogings en mag verantwoordelik wees vir die verskillende vlakke van sukses wat waargeneem is. Alhoewel daar wel genetiese diversiteit binne populasies van die predator waargeneem is, is verdere studies nodig om die bydrae van hierdie diversiteit tot die effektiwiteit van beheer onder veldtoestande te bepaal. Die effek van laboratorium-geteelde kolonies in die toename in genetiese drywing moet geakkomodeer word ten einde ’n verlies aan diversiteit en aanpassing by teelomstadighede te verhoed.

Sleutelwoorde: *Teretrius nigrescens*, *Prostephanus truncatus*, genetiese differensiasie, molekulêre merkers, DNA volgorde-analises, mikrosatelliete, ekologiese volhoubaarheid.

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CHAPTER ONE

General Introduction and Literature Review

1.1 Introduction

The larger grain borer (LGB) *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) is the worst pest of farm-stored maize and cassava in Africa today (Schneider *et al*., 2004). It was accidentally introduced into the continent in the 1970s and has since spread to at least 18 countries (Schneider *et al*., 2004; Gueye *et al*., 2008). The LGB causes a mean weight loss of about 34 % within the first six months of grain storage, five times that caused by all the indigenous storage pest fauna (Hodges *et al*., 1983, Giles *et al*., 1996). This damage is particularly costly as it occurs when all the production costs have been incurred and when plant compensatory growth is not possible. Economic losses due to poor product marketability, pricing, seed viability and possible association with post-damage contamination can lead to total economic loss.

In Kenya, annual losses were estimated at 730,000 tonnes, valued at KShs. 8.1 billion (US $ 108 million at current exchange rates (www.xe.com, March, 14, 2009) (Anonymous, 2003). The larger grain borer spread into the country from Tanzania and initially established in the semi-arid grain deficient coastal and eastern region (Hodges *et al*., 1998). Strict containment efforts were imposed on the movement of maize within Kenya, to prevent *P. truncatus* spreading to the main maize-surplus areas in the west of the country (Hodges *et al*., 1998). The pest has recently been reported in the cool high production highlands like the western Rift Valley in Kenya. This expansion poses the threats of both direct damage of bulk-stored grain and rapid dispersal from these maize-surplus regions during grain movement. It is also a new impediment in the grain industry, food security and the contribution of agriculture to the country's GDP.

The establishment of the LGB in Africa has also changed the post-harvest pest control paradigm. Storage of unshelled maize was hitherto effective against native post-harvest pests like the maize weevil, *Sitophilus zeamais* (Motchulsky) (Coleoptera: Curculionidae) and Angoumis grain moth *Sitotroga cerealella* Olivier (Lepidoptera: Gelechiidae), but facilitates infestation of maize by the LGB. Good store hygiene, cob segregation and sun-drying have been used in the traditional systems, but are not sufficient against the LGB (Giles *et al*., 1996). Although shelling maize significantly reduces damage by the LGB (Cowley *et al*., 1980), storage in sacks stabilises it enough to encourage attack. Treating shelled grain with binary pyrethroid-organophosphate insecticide dusts (such as Actellic Super) has been useful in pre-infestation protection. Recently, a spinosad-based insecticide has been introduced against storage insects (Fang *et al*., 2002). Chemical control is not technically sustainable in the subsistence storage systems due to the possibility of using inappropriate toxicant doses, counterfeit and diluted pesticides or development of resistance to pesticides. In high LGB risk areas, many farmers evade losses by disposing of their produce at low prices soon after harvest, forcing them to buy grain at higher prices later in the season (Meikle *et al*., 2000; Hodges, 2002).

The presence of the LGB populations outside storage environments is a challenge to conventional control measures. *Prostephanus truncatus* is not exclusively a post-harvest agricultural pest but a woodborer, which has adapted to dried stored products. It can survive on several species of trees in Africa (Nang'ayo *et al*., 1993; Nansen *et al*., 2004). Cultural and chemical measures would be impractical or too expensive in the wild habitats. Adapted natural enemies that can locate the pest outside the agricultural ecosystem could help reduce the rate of re-infestation of disinfected stores and the early season pest pressure (Nansen and Meikle, 2002).

Teretrius (formerly *Teretriosoma*) *nigrescens* Lewis (Coleoptera: Histeridae), a predatory beetle, found to be consistently associated with *P. truncatus* in the native range, has been introduced into Africa in a classical biological control approach (Hoppe, 1986; Böye *et al*., 1988; Borgemeister *et al*., 2001). This egg and larval predator has specific preference for *P. truncatus* (Ayertey *et al*., 1999; Rees, 1991) and cues in on the pest's aggregation pheromone, as a kairomone, to locate its prey (Rees *et al*., 1990; Scholz *et al*., 1998). It is also able to disperse fast in the wild on its own (Borgemeister *et al*., 1997a).

Biological control of the LGB using *T. nigrescens* has been carried out in several African countries with variable levels of success. The release of a Costa Rican population of the predator resulted in lower LGB flight activity and a reduction of grain damage trends in warm humid coastal areas of Togo, Benin and Ghana (Borgemeister *et al*., 1997a). An 80 % reduction in LGB flight activity and 70km dispersal was observed within three years of release of a Mexican population in Kenya (Hill *et al*., 2003). Yet, while control in coastal West Africa was sustained over 10 years, that in eastern Africa has largely collapsed. In Guinea Conakry, the Benin population failed to establish under cool highland conditions, comparable to the failure of the predator to pursue the LGB beyond the semi arid eastern region of Kenya. Climate and strain characteristics, therefore, determine the success of this approach. Cold and dry climates limit the performance of *T. nigrescens* more than that of *P. truncatus* (Tigar *et al*., 1994).

Population differentiation and ecological and climatic limitations of the predator are not fully understood. Tigar *et al*. (1994) recovered it from highland areas of La Laguna and Texcoco in Mexico, while Rees (1990) observed the predator in very dry coastal areas near Yucatan Peninsula. Weather conditions of these areas are comparable to those in either cool humid Highlands or the dry Sahel, both of which have failed to sustain the establishment of its populations. Although intraspecific variation has been reported in the LGB (Guntrip *et al*., 1996; Marshed-Kharusy and Dawah, 1999) it does not significantly affect predation by *T. nigrescens* (Bergvinson, CiMMYT, Mexico, personal communication). It is therefore likely that predator population characteristics and their adaptation to environmental conditions play a key role in the levels of success observed in the biological control of the LGB in Africa.

Six new populations of *T. nigrescens* recovered from various areas in Mexico and Honduras and two populations from Mexico and Costa Rica earlier released in Africa, are being studied under quarantine at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, for possible use in LGB control programmes in eastern and southern Africa. Their release must be preceded by the development of reliable means of monitoring the strains and their biological interactions with each other, the pest and the environment. Characterisation of the strains of *T. nigrescens* is essential in quantifying, assessing and interpreting the performance of the strains of *T. nigrescens* used. Since biological differences have been noted without associated morphological differences between the putative biotypes, unequivocal differentiation using molecular markers is invaluable in this programme.

This study, therefore, sought to answer the following questions: what is the level of genetic differentiation within *T. nigrescens*? Do ecotypes of the predator exist? Do *T. nigrescens* geographic populations or putative strains have distinct preferences to measurable environmental conditions? Can the putative strains be discerned using simple molecular markers? How can diversity within *T. nigrescens* be harnessed for sustainable control of the LGB in Africa?

Consequently, baseline monitoring of *P. truncatus* and *T. nigrescens* was done to assess the current distribution and flight activity of the two species in Kenya. Putative geographic strains of the predator were compared to for their preference to temperature and humidity regimes. Mitochondrial and nuclear genetic markers were used to trace the phylogenetic history of the populations and to apportion its variation. Novel microsatellite markers were developed and utilised to assess the population genetic structure and demographic history of the predator. Finally, a simple molecular procedure has been proposed for differentiating four populations.

1.2 General Literature Review

1.2.1 Maize and cassava in Africa

Maize and cassava are the most important staple food crops in Africa. The acreage of maize in the continent is about 26 million hectares, with an annual production of 42 million tonnes. In Kenya, 2.3 million tonnes are produced on some 1.5 million hectares (FAO, 2004). Over 103 million tonnes of cassava is produced on the continent almost exclusively by subsistence farmers (FAO, 2004). Because of its drought tolerance, cassava is an important food security crop to about 20 million Africans (Legg, 1994). Significant failure of the two commodities therefore threatens food security and economic stability of the poor communities least able to bear the burden.

Production of the two crops remains low, mainly due to climatic constraints and biotic factors. Field pests such as lepidopteran stem borers and diseases, for example, the maize streak virus, downy mildew and grey leaf spot, are major constraints to maize production. Similarly, the cassava mosaic geminiviruses seriously threaten yield stability of cassava (Legg *et al*., 1994). Post-harvest losses are therefore especially painful since they occur after all production costs have been incurred. In developing countries, about 90 % of cereals produced are intended for human consumption, so, improved post-harvest protection measures could increase food supply by 30 – 40 % (Pantenius, 1988).

1.3 Post-harvest losses

The LGB causes multi-dimensional and very costly damage to grains. Losses of quality, grain weight and those due to forgone income or extra expenditure in grain protection efforts can total to over 50 % of the loss in product value (Magrath *et al*., 1996) (Figures 1.1, 1.2, 1.3). Mean grain losses in Africa have been estimated at between 9 % and 13 % in the first six months (Golob, 1988; Pantenius, 1988). Insects account for 80-90 % of this loss (Pantenius, 1988). The lower yielding indigenous landraces of maize generally suffer lower post-harvest losses than the high yielding improved varieties (Prevett, 1990).

The level of post-harvest grain losses in sub-Saharan Africa increased dramatically following the introduction and establishment of the LGB in the late 1970s and 1980s and its subsequent spread (Farrel and Schulten, 2002). Reported loss estimates include 8 % per month in Togo; 30 – 50 % on unshelled maize in Burundi and up to 34 % in Kenya (Pantenius, 1988; Nang'ayo, 1996). The presence of the LGB also complicates food supply to grain deficit areas where containment approaches are effected. Yet farmers shell and sell maize early in the season in most affected areas to avoid rapid damage by the pest. Early disposal of surplus maize and repurchase during deficit seasons leads to double loss of selling at low prices and later buying the same produce (usually of a lower quality) at higher prices.

Figure 1.1: The Larger Grain Borer, *Prostephanus truncatus* (courtesy of G. Goergen, IITA)

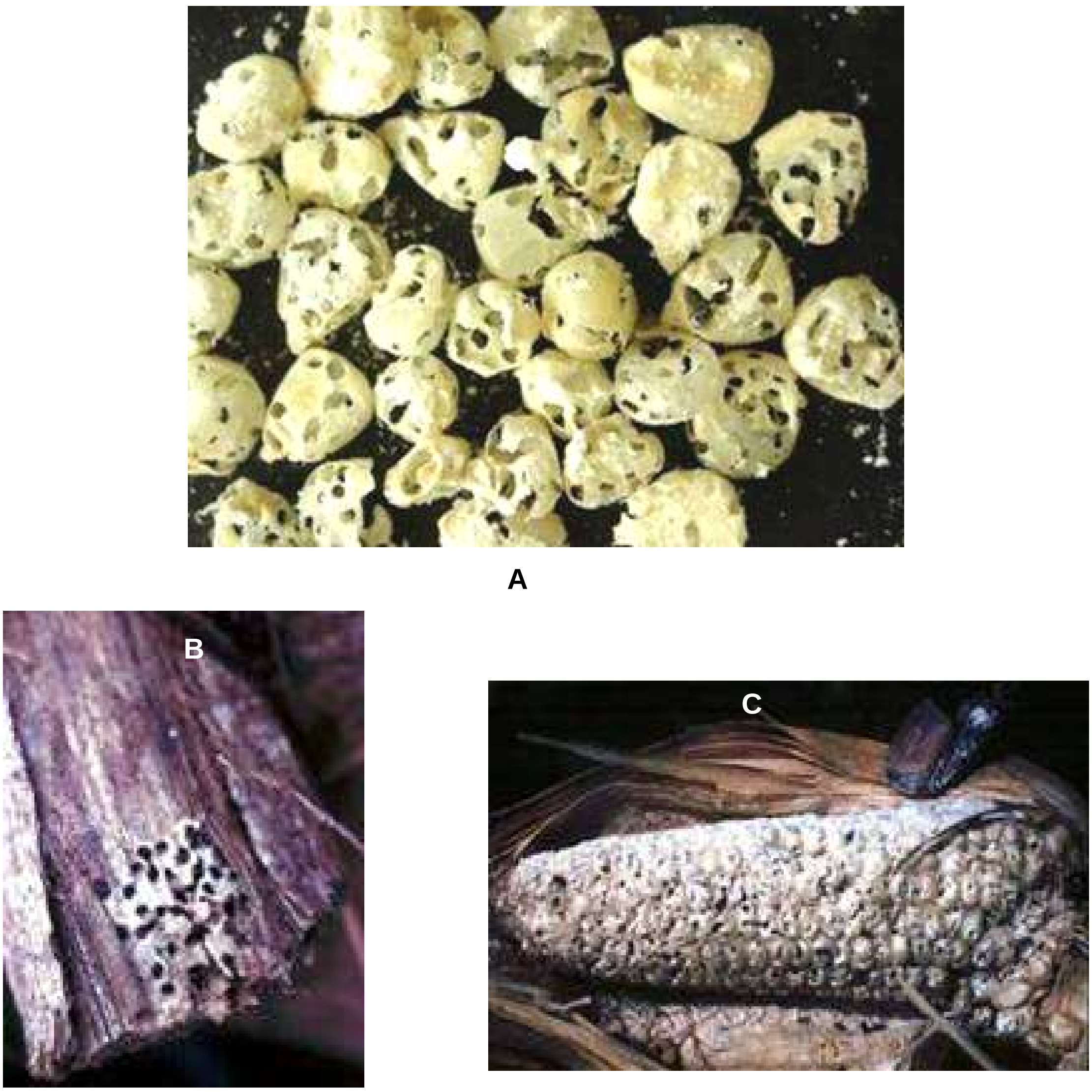

Figure 1.2: LGB damage on different substrates: **A-** maize kernels, **B-** wood and **C**-cob of maize (pictures B and C: courtesy of C. Borgemeister, ICIPE). LGB frass was cleaned off substrates to expose damage.

1.3.1 *Prostephanus truncatus* in Africa

The LGB is a native of Central America where it has long been recognised as an occasional pest of stored maize (Boxall, 2002). It was accidentally introduced into Africa in the 1970s (Dunstan and Magazini, 1981; Krall, 1984) and has now spread to at least 18 countries (Schneider *et al*., 2004; Gueye *et al*., 2008). The LGB causes rapid deterioration in the mass and quality of maize, dried cassava chips and even wood (Borgemeister *et al*., 2001; Boxall, 2002) (Figure 1.1). Its introduction and spread has been attributed to movement of grain in trade and distribution of relief food. In fact, heavy infestations are often recorded near maize transit routes and grain markets (Tyler and Hodges, 2002).

The LGB is a polyphagous insect, surviving and reproducing on agricultural and non-agricultural substrates. It mainly infests maize and cassava but also wheat, paddy pulses, groundnuts, cocoa and coffee beans and dry tubers of yam and sweet potato (Shires, 1977; Boxall, 2002). It can also infest above-ground wood, woody roots and seeds of several tree species (Nang'ayo *et al*., 2002; Nansen *et al*., 2004). In Africa, high trap catches have been recorded in forest environments, away from any farmlands, grain storage or market areas, underlining the importance of the natural non-agricultural habitats in *P. truncatus* ecology (Hill *et al*., 2003). Woody tree branches girdled by cerambicid beetles are, so far, the only non-agricultural hosts in which the LGB adults and larvae have been found in the forest (Nansen *et al*., 2004). Perhaps, such hosts play an important role in maintaining populations in the off-season before other food substances become available (Nang'ayo *et al*., 1993; Ramirez-Martinez *et al*., 1994).

1.4 Monitoring and detection of stored product pests

Surveys of post-harvest pests generally focuses on detection and monitoring populations. Detection aims at finding the presence of the pest, especially to support early warning systems or to aid to control decision making (Likhayo and Hodges, 2000; Hodges, 2002). Conversely, monitoring attempts to estimate changes of pest numbers over time. Early detection of storage pests is important since they uniquely infest the portion of the plant of the highest quality, and also at a time when compensatory growth is no longer possible. Losses cannot be made up for and are relatively economically more costly compared to early-season foliage damage. Many post harvest pests are cryptic and, therefore, detection of infestation is more critical than the estimation of population size since the latter is often difficult to determine (Hodges, 2002; Throne *et al.*, 2003; Xing and Guyer, 2008). Since storage structures generally do not permit individual inspection of stored units, pest detection is invaluable. Data from pest monitoring are useful in detecting trends over time which would be associated with specific environmental conditions (Hodges, 2002).

The methods of pest monitoring and detection vary from physical inspection of samples, transmittance spectroscopy and pheromone luring both inside and outside of the storage environments (Hodges, 2002; Xing and Guyer, 2008). For the LGB, traps are generally more effective than physical inspection. Probe traps are used for sampling insects moving through grain and can be baited to increase their attractiveness (reviewed by Hodge, 2002 and Throne *et al.*, 2003). However, these traps require entering into the storage facility and are labour intensive. Also they are not useful for detecting insect stadia feeding and developing inside grains. Flight traps have been used for pest monitoring in the grain headspace and outside the store. Pheromone baited flight traps are in use for detecting *Sitophilus zeamais* (Dendy *et al.*, 1991; Likhayo and Hodges, 2000). The design of such traps depends on a clear understanding of the pest biology and ecology and an understanding of their working.

1.4.1 Monitoring *P. truncatus*

Probe and pheromone-baited crevice traps were initially used for LGB monitoring in grain stores (Dendy *et al*., 1991). However these traps could detect the pest in only one third of the infestations in Mexico (Rees *et al*., 1990). This is perhaps because the aggregation pheromones are much more attractive to migrating insects looking for feeding substrates (Fadamiro and Wyatt, 1995). The LGB have also been caught in unbaited light traps, but these catch many species of insects and catch identification may be difficult. Similarly, they are not specifically attractive to the pest species. Flight traps are now the standard monitoring tool for LGB detection. The design of flight traps influences their attractiveness to *P. truncatus*. Delta or wing traps are not as attractive as funnel traps but much easier to deploy and not as prone to vandalism. Funnel traps are more protected from saturation, allow for addition of arrestants for trapped animals and are more consistent. They are therefore more useful for live recovery of samples (Hodges, 2002).

The interpretation of flight trap data is critical. Flight traps generally catch insects in the dispersal phase and (for the LGB) are more attractive to young mated females (Hodges, 2002). The LGB aggregation pheromone orientates flying insects towards the pheromone source but does not induce flight (Fadamiro and Wyatt, 1995). Ambient temperature during development, food quality and other environmental triggers of flight have all been shown to contribute to dispersal (Fadamiro and Wyatt, 1995; Scholz *et al*., 1997; Borgemeister *et al*., 1997b; Hodges *et al*., 2003). Instantaneous flight trap catches are therefore an indication of the propensity of the pest to disperse, but may only give an indication of the actual available population if long term trends are considered and accounted for (Hodges, 2002).

1.5 Control of stored product pests

Cultural, physical and chemical means are used to manage post harvest pests in Africa. Maintenance of hygiene by complete emptying of and cleaning of stores before re-stocking reduces pest carry over between seasons. Sun drying of grains and treatment with inert dusts and botanical products are common in subsistence farming systems (Giles *et al*., 1996). These methods are effective against most post-harvest pests but do not ensure adequate control of the LGB. For instance, maize storage in husks or on cobs discourages infestation by most weevils and grain moths, but promotes infestation by the LGB (Giles *et al*., 1991; Ayertey *et al*., 1999). Shelling maize before storage and treating it with insecticides have been effective against indigenous post-harvest pests and the LGB in parts of East Africa, but not in West Africa (Ayertey *et al*., 1999).

1.5.1 Control of the larger grain borer

Methods of control of the LGB have evolved as more knowledge of the pest becomes available (Farrel and Schulten, 2002). Containment by exclusion and eradication was the first approach attempted, but was technically and logistically difficult to implement (Hodges, 2002). Eradication of the pest succeeded in Israel and Arabia but not in Africa and integrated approaches were proposed (Farrell and Schulten, 2002).

Both preventive and curative control measures have been used. Restriction of grain movement from and through infested regions has been attempted, but has been difficult to enforce, since cross-border smuggling and informal movement of grain is common (Golob, 2002). Such activities, perhaps, introduced the pest into Burundi from Tanzania (Farrell and Schulten, 2002). Chemical treatment in farm stores and natural habitat has been attempted. Fumigation of stored produce using methyl bromide and aluminium phosphide (phostoxin) has been effective (Golob, 2002) although it is technically and logistically impractical at small farm level. Contact and stomach insecticides, spread in inert powder or as liquids, protect the grain surface, and are therefore more potent on shelled maize or when used before infestation. Once cobs are attacked, adult LGB adults live and reproduce inside grain and might escape control (Golob, 2002). Use of pesticides is also limited by knowledge levels, hence the possibility of using fake and diluted products and incorrect doses. The effectiveness of a combination of organophosphates and pyrethroids is reducing as the pests become resistant (Golob, 2002). New pesticides based on Spinosad have been introduced in the market (Fang *et al*., 2002). Spinosyns are fungal secondary metabolites, so, the development of resistance is likely in the long run. An integrated management approach including the storage technology, hygiene, post-harvest treatment, biological control and supported by proper monitoring tools are presently favoured (Farrell and Schulten, 2002).

1.5.2 Biological control

Prostephanus truncatus is an introduced species and only of minor pest importance in its area of origin (Pantenius, 1988). Practical challenges to chemical control rendered it an ideal candidate for classical biological control (Schultz and Laborius, 1987, 1988). Two Protozoa, *Mattesia* and *Nosema*, were not effective under semi-field trials while two strains of bacteria caused significant mortality in the laboratory but were ineffectual in field studies (Poshko, 1994). Isolates of the fungus *Metarrhizium anisopiale* (Metchinkoff) Sorokin were highly virulent but their use was not pursued due to technical and safety reasons (Schulz and Meikle *et al*., 2002).

Of the natural enemies recovered, *T. nigrescens* was the most consistently associated with the pest and effective in causing significant mortality in the laboratory and in the field (Böye, 1988). It was also significantly coadapted to predation on the LGB. The predator was therefore released in several areas in Africa between 1991 and 1996 for the control of the

LGB (Meikle *et al.,* 2002). Several studies have reported reduction in flight activity, damage level and infestation rates (Borgemeister *et al*., 2001; Farrel and Schulten, 2002; Hill *et al*., 2003). Other workers argue that the sustainable successful use of *T. nigrescens* is doubtful, and fluctuations in flight activity were mainly due to climatic conditions, hence clear understanding of the post-harvest system is a prerequisite to its use (Holst and Meikle, 2002; Meikle *et al*., 2002). Integrated management approaches should include knowledge of the ecological limitations of the pest and predator and development of effective monitoring and warning systems (Boxall, 2002; Farrel and Schulten, 2002).

1.6 *Teretrius nigrescens*

Teretrius nigrescens Lewis belongs to the family Histeridae as do over 3700 other species (Mazur, 1997). They are tiny beetles, 2-3mm long with a compact body (Figure 1.3) (Poshko, 1994). Histerids are predators of necrophagous and wood boring insects and mites. Most histerids are either oligophagous or polyphagous living in carcasses and leaf litter (Dégallier and Gomy, 1983; Stewart-Jones *et al*., 2004).

Teretrius nigrescens shows a distinct preference for *P. truncatus* (Poshko, 1994). Although it can also prey upon *Dinoderus* spp., *Rhizopertha dominica* (Fabricius) (Coleoptera: Bostrichidae), *Sitophilus* spp. (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), which are common storage pests of cereals in Africa (Rees, 1987; 1990; Poshko *et al*., 1992; Ayertey *et al*., 1999). Its performance on these species is however very poor. Studies showed that *T. nigrescens* does not pose a threat to the local beneficial insect fauna due to its prey finding strategy and distinct prey preference (Farrel and Schulten, 2002). In stores, it survives on grain without the prey for long periods, but reproduction would be suppressed under these conditions.

Figure 1.3: Adult *Teretrius nigrescens* (courtesy: G. Goergen, IITA, Benin).

The bar represents 1 mm.

Teretrius nigrescens is well adapted to the habitat and habits of its prey. The body size and fossorial adaptation of the fore tibia of the adults enable it to pursue the borer into the substrate. The larvae are voracious predators of the eggs and larvae of the LGB. Behaviourally, the predator exhibits strong attraction to the aggregation pheromones of *P. truncatus* (Scholz *et al*., 1998) and non-volatile compound(s) in the dust of the LGB (Rees, 1990; Stewart-Jones *et al*., 2004). In Mexico and Costa Rica, it was only observed in association with the LGB, both in natural environments and maize stores (Böye, 1988; Rees, 1990).

The predatory capacity of *T. nigrescens* and its ability to control the LGB have been widely studied under laboratory and field conditions. Both larvae and adults prey on *P. truncatus* eggs and larvae (Rees, 1985; Poshko, 1994). A single larva consumes about 60 LGB larvae (about 5 daily) to develop to an imago, while an adult kills about 1.1 larvae a day (Böye, 1988). An 80 % decline in the LGB population has been recorded in laboratory bioassays. In the field, the predator disperses faster than its prey *P. truncatus* (Borgemeister *et al*., 1997a). Field releases of *T. nigrescens* have resulted in a reduction in losses, maize damage, LGB flight activity and infestation levels (Giles *et al*., 1996; Borgemeister *et al*., 2001; Hill *et al*., 2003; Schneider *et al*., 2004). The performance of *T. nigrescens* as a biological control agent depends on several environmental factors. Grain damage and weight loss are usually lower and suppression of LGB population growth better on shelled than cob-stored maize (Poshko, 1994). There is little evidence however of control of the LGB by *T. nigrescens* on cassava, yam and other bulk substrates.

Few studies have conclusively reported the effects of climate on the development or performance of *T. nigrescens* in the control of the LGB. Anecdotal observations indicate that *T. nigrescens* is more limited by cool temperatures than its prey *P. truncatus*. Oussou *et al*.

(1998) did not establish any relationship between relative humidity conditions and larval survival, although 30 % RH appeared marginally more suitable than higher and lower humidity conditions. Leliveldt (1990) observed higher predatory ability of *T. nigrescens* at 30 ^{o}C than at 26 ^{o}C on *P. truncatus* reared on maize. The recent reports of the pest dispersal to cool maize surplus areas especially in Kenya are, therefore, a major concern, as the ecological limitations of the *T. nigrescens* strains are not clearly understood.

1.7 Molecular methods in insect population genetics

Several molecular markers are available for the study of inter- and intra-specific variation and genetic structure of insect populations. These have been variously applied to delineate species, determine insect phylogenetic relationships (Caterino and Vogler, 2002) and elucidate intraspecific variation (Omondi *et al.*, 2004; Berry *et al.*, 2004). Caterino and Vogler (2002) used the 18S rDNA sequences in combination with morphological characteristics to resolve the phylogeny of the superfamily Histeroidea. However, each marker system comes with a set of assumptions, strengths and limitations restricting the ecological question it can be used to address (reviews by Loxdale and Lushai, 1998; Avise, 2000; Estoup *et al*., 2002; Zhang and Hewitt, 2002).

Within *T. nigrescens*, no reports of DNA based markers available. Molecular markers for such exploratory studies should be those that either require no prior sequence information for the species (e.g. RAPD-PCR, AFLP) or those for which probes may be obtained from other closely related studied species such as sequence analysis of a conserved gene.

1.7.1 **RAPD-PCR**

Randomly Amplified Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR) technique characterises DNA by amplifying fragments whose location is priorly unknown.

Short (8 – 10 bp) primers of arbitrary sequence, which anneal to several locations on the genome, are used (Welsh *et al*., 1990; Williams *et al*., 1990). The products are then separated by electrophoresis and a comparison based on the extent of band sharing between pairs of individuals.

RAPD-PCR technique is likely to sample the genome more randomly, does not require prior sequence information on the subject species and utilises available random primers across species (Lynch and Milligan, 1994). It is also technically simple. The results however are usually not reproducible between laboratories, experimental protocols and time. Also comigration of equal sized fragments of different sources may exaggerate the similarity signal (Lynch and Milligan, 1994). Being a dominant marker it is limited on the potential number of alleles for the comparison of samples. Practical procedures are available for the improvement of the reliability of this marker (Gawell and Bartlett, 1993; Lima *et al*., 2002). RAPD-PCR has been useful in studies of population structure of insect species (Hadrys *et al*., 1992; Cenis *et al*., 1993; Lima *et al*., 2002; Omondi *et al*., 2004) identification of insect biotypes (De Barro and Driver, 1997; Moya *et al*., 2001) mating studies (de Barro and Hart, 2000; Omondi *et al*., 2005). Sequencing unique RAPD bands has also led to the development of sequence characterised amplified regions (SCARs) markers (Rugienius *et al*., 2006).

1.7.2 Amplified fragment length polymorphism

Amplified (restriction) fragment length polymorphism (AFLP) technique combines the specificity of restriction enzyme digestion with the reliability of PCR to detect DNA polymorphism. It is based on the selective amplification of a fraction of the fragments obtained after DNA restriction, allowing high resolution of genetic differences. These markers are dominant as they display the presence or absence of restriction fragments and not their length differences. In entomological investigations, AFLP has been used to study genetic variation among whiteflies (Cervera, 2000), linkage mapping in moths (Heckel *et al*., 1998) and fruitfly taxonomy (Kibogo, 2005). It is therefore of potential utility in the design of group specific primers for molecular diagnosis, if any Sequence Tagged Sites (STSs) can be recovered and sequenced (Brugmans *et al*., 2003). It is a method of choice for its repeatability and robustness while using arbitrary primers.

1.7.3 Mitochondrial DNA markers

Mitochondrial genomes are a rich source of markers for studies of population genetics and evolution. In animals, the mitochondrial genome is generally circular (4-17kb) is maternally inherited and has a relatively simple genetic structure and high mutation rates (Adams and Palmer, 2003; Thao *et al*., 2004). The DNA molecule is made up of 37 genes coding for 22 transfer RNAs, two ribosomal RNAs and 13 messenger RNAs and generally lacks introns. The rDNA on the other hand has large families of repetitive DNA, pseudogenes and large spacer sequences (Loxdale and Lushai, 1998). Within the class Insecta, the order of mitochondrial genes is highly conserved leading to the proposal of an ancestral gene array (Shao and Baker, 2003).

Different gene regions of the mtDNA have been found to change at different rates (Lunt *et al*., 1996). The control region changes more rapidly both within and between species, whereas the ribosomal RNA genes (e.g. 12S and 16S) evolve more slowly (Moritz, 1987; McPheron and Han, 1997). Mitochondrial DNA (mtDNA) is therefore used for taxonomic and population genetic studies of insects (Loxdale and Lushai, 1998). Since there are more mitochondria compared to nuclei in a cell, there are more copies of mtDNA than those of nuclear DNA hence mtDNA can be detected in old and minute and physically degraded samples. The mtDNA is probably the most widely used source of DNA for molecular investigations and a good starting point for preliminary investigations (Zhang and Hewitt, 2003).

Universal PCR primers have been described to amplify across different sections of the mtDNA (Zhang and Hewitt, 1997; Loxdale and Lushai, 1998). Divergence of nucleotide sequences of various mtDNA genes and subsequent sequence alignment to reveal nucleotide differences has been used to designate insect biotypes, notably the use of the mtCOI gene in *Bemisia tabaci* biotype designation (Berry *et al*., 2004). A 688 bp region of the 5′ end mtCOI gene has been proposed as a possible marker evolving at the rate of speciation in animals and is the gene of choice for the genetic barcoding and identification of animal species (Hebert *et al.*, 2003; Barrett and Hebert, 2005).

1.7.4 Nuclear DNA markers

The use of nuclear genetic markers in population genetics has been reviewed by Zhang and Hewitt (2002). Nuclear sequences have an advantage over mitochondrial genomes because they are inherited from both parents, hence can be transferred across species and population boundaries in hybrid zones (Zhang and Hewitt, 2003). However, recombination may distort the genetic content in nuclear makers leading to a false inference of genealogy (Zhang and Hewitt, 2003). For specific ecological studies, the choice of the marker to use is critical. Expressed (or coding) sequences are often more stable and useful for studying higher taxonomy differences since they have greater fitness costs to possible mutational changes. Conversely, introns may accumulate a greater extent of changes without a fitness cost and so find practical application in population level studies (e.g. Fritz *et al*., 1994; Gomez-Zurita *et al*., 2000).

Ribosomal genomes provide a uniquely powerful source of genomic information. The ribosomal genome consists of tandem repeats of exons, introns and internal transcribed spacers that are transcribed into RNA but not expressed into protein. These segments of DNA are widely used as data can be obtained from both sequence and secondary structure characteristics. Although inherited from both parents, concerted evolution events tend to fix changes within genomes and populations making them useful for studying population boundaries (Zhang and Hewitt, 2003). The structure and sequence of coding regions of rDNA are well conserved, while spacers are highly variable. Therefore, primers developed from the conserved region in one species can be used to amplify the variable regions for fine-scale population studies in an even remotely related species (Fritz *et al*., 1994). Similarly, sequences of the conserved regions are useful for studying deep phylogenetic relationships (e.g. Caterino and Vogler, 2002).

1.7.5 Microsatellite markers

Microsatellite sequences are short core (2-10bp) tandem repeats up to 500 bp long scattered throughout the genome (Weber and May, 1989). They mainly occur in non-coding DNA, closely associated with conserved loci (Ellegren, 2004). These markers are co-dominant and hyper-variable and their inheritance can be determined by simple mating studies. The level of genetic variability produced has made these markers, the most popular in ecological genetics studies. Microsatellites are useful Mendelian markers, with a fairly simple modes of evolution (Estoup *et al*., 1995; Ellegren, 2004) and are useful in population genetics, parentage studies and reconstruction of recent demographic events (Selkoe and Toonen, 2006).

An important set back in the use of microsatellites is the requirement of *de novo* isolation of new markers if they are not yet available for a particular species or its congeneric relatives. Since each marker isolated comprises just a single data point, several markers must be isolated and tested for use in population genetic studies (Zane *et al.*, 2002; Selkoe and Toonen, 2006). However, once primers have been developed, they may be used in closely related taxa (Ellegren, 2004). Advances in sequencing technologies (Hudson, 2008) have significantly reduced the technical effort and cost of required in isolating new microsatellites markers (Zane *et al.*, 2002; Selkoe and Toonen, 2006; Santana *et al*., 2009).

1.8 Population genetics in biological control

The availability of genetic tools for population genetics has increased the precision with which we can now study the interactions between crops, pests and their natural enemies in diverse environmental conditions. Roderick and Navajas (2003) have reviewed the utility of genetic information in biological control. Biological control would benefit from an accurate identification of the geographic source of an invasive species, tracking of genetic changes in populations since introduction and guided maintenance of genetic diversity of natural enemies. Molecular markers have been used to assess the genetics of population establishment, audit and improvement of approaches to biological introductions (Omwega and Overholt, 1996) and to study the basis of pest-natural enemy interactions (Gitau *et al*., 2007). It is appropriate to focus on the source population of the invader to recover closely coadapted populations of natural enemies. Similarly, bioprospecting for natural enemies would be improved by ecological matching and genetic typing of ecological populations of the natural enemies to improve establishment in the areas of introduction. The chances of success may also be increased through breeding and genetic modification for better establishment and survival of the natural enemies, their effectiveness (especially pathogens) and ability to locate their hosts (Handler and Beeman, 2003). Advances in molecular biology, the development of multilocus markers and sequence data as well as analysis protocols has improved our ability to detect the genealogical origin, population history and underlying genetic interactions of populations and species. Similarly, the information of insect species genome sequencing projects, including *Drosophila melanogaster* (Diptera; Drosophilidae), *Apis mellifera* (Hynemoptera: Apidae), *Bombyx mori* (**Lepidoptera**: Bombycidae), *Anopheles gambiae* (Diptera: Culicidae) and the post harvest pest *Tribolium castaneum* (Coleoptera: Tenebrionidae), have availed the available information for the genetic manipulation and monitoring of pests and their natural enemies.

The effectiveness of biological depends on the establishment and adaptation of the introduced natural enemies to exploit the pest in the new environment (Roderick and Navajas, 2003). If adaptation of well characterised natural enemies is important, then it is essential to preserve a high level of genetic diversity in introduced natural enemies too (Roderick and Navajas, 2003). Establishment of an introduced population is often correlated with the propagule pressure (also called introduction effort) which is a product of the introduced numbers (propagule size) and the genetic diversity (Blackburn and Duncan, 2001; Lockwood *et al*., 2005; Bacigalupe, 2008). Rapid adaptation of natural enemies has been shown for microorganisms but less for insect predators and parasitoids (Holt and Hochberg, 1997). Such adaptation depends on the availability underlying genetic diversity upon which natural selection in the new environment would act (Lockwood *et al*., 2007; Bacigalupe, 2008). A geographical analysis of the genetic architecture of the populations of species would therefore provide important information in facilitating biological control efforts.

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CHAPTER TWO

General Materials and Methods

2.1 Study materials

2.1.1 Insects

Samples of *Prostephanus truncatus* collected from maize stores and the field in Kenya were used to establish cultures on maize based on the region of origin at the Animal Rearing and Quarantine Unit (ARQU) at ICIPE. Starter colonies of *Teretrius nigrescens* were obtained as outlined in Table 2.1. The KARI (Kenya Agricultural Research Institute) strain was subcultured from the remnant colony of the *T. nigrescens* originally coming from Mexico, released in Kenya in 1992 and now maintained at KARI, Kiboko Field Station (Giles *et al*., 1996). The Benin strain was obtained from International Institute of Tropical Agriculture (IITA), Abomey-Calavi, Benin. This population had been established from adults recovered from granaries in Benin, three years after field releases in Togo (C. Atcha, WARDA, personal communication). The Togo releases had been recovered from Sardinal, Guanacaste, north-western Costa Rica (Böye, 1990). These populations were assumed to be descendants of insects that had been released in Kenya, Benin and Togo, respectively, in biological control efforts between 1990 and 2000. Three geographical strains from colonies established using insects recovered from granaries at El Batan (hereafter referred to as the Batan strain), Oaxaca and Tlatizaspan in Mexico were imported from CiMMYT, Mexico. Three other populations were collected from Teupasenti, Gualaso and Yoro in Honduras between 2006 – 2007 and used to found laboratory cultures. The Kiboko colony was established using insects recovered in 2007 from the Kiboki Range, eastern Kenya within the original outbreak and biocontol area (Giles *et al*., 1996). The ‘Store’ colony was accidentally found in LGB-infested wooden pegs from a store in ICIPE. These pegs had reportedly been used for field sampling cereal stem borers and their parasitoids a year earlier. The origin of this population was unknown. All *T. nigrescens* strains were reared on *P. truncatus* raised on maize at the Animal Rearing and Quarantine Unit (ARQU) at ICIPE, Duduville, Nairobi, Kenya. The LGB and *T. nigrescens* were reared using a modification of protocols described by Giles *et al*. (1996) and Atcha and Borgemeister (unpublished IITA manual).

Three populations of alcohol-preserved samples were also used. The Ghana strain was obtained from a colony maintained at the Natural Resources Institute (NRI), UK, established with insects initially recovered from the field in Ghana in 1998 following releases in Togo, Benin and possibly Volta Region, Ghana (R. Hodges, NRI, personal communication). Malawi samples, originated from a colony maintained at the Crop Protection Agency, Ministry of Agriculture, Malawi. This colony was a subculture of the Benin colony (Anonymous, 1999). The Mombasa population comprised of five individuals caught on a pheromone-baited sticky trap at Mombasa. These traps were set after the discovery of the "Store" population, since the substrate material was it was discovered in was had been reportedly used in this region.

Table 2.1: Field sources and culturing history of the populations *T. nigrescens* used in this study

Name	Field Origin	Type	Date sampled	Rearing history	Remarks
KARI	Neuvo Leone, Mexico	Laboratory colony	1990	KARI colony, 16 years	Earlier released for BC in Kenya
Benin	Guanacaste, Costa Rica	Laboratory population	1989	IITA colony 18 years	Established from field samples collected in Benin
Ghana[1]	Ghana	Laboratory colony	1989	NRI colony from field samples for 10 year culturing	Possible progeny of Benin samples
Teupasenti	Honduras	Directly recovered from the field	2007	Laboratory culturing for 1 year	Originating from south western Honduras
Gualaso	Honduras	Directly recovered from the field	2007	Laboratory culturing for 1 year	Mid altitude Honduras population
Yoro	Honduras	Directly recovered from the field	2007	Laboratory culturing for 1 year	High altitude Central Honduras population
Oaxaca	Mexico	Young laboratory colony	2003	CiMMYT colony, 3 years	Mid altitude population, southern Mexico
El Batan	Mexico	Young laboratory colony	2003	CiMMYT rearing for 3 years	Low altitude population Central Mexico
Tlaltizaspan	Mexico	Young laboratory colony	2003	CiMMYT rearing for 3 years	High altitude population, upper central Mexico
Malawi[1]	Costa Rica	Laboratory colony	1990	From Benin colony, rearing for 9 years	Sub-culture of Benin colony
Store	Unknown (Kenya)	Recovered from LGB-infested wood in Project Store	2006	1 year	Antiquity unknown, Possible descendants of KARI releases)
Field	Kenya	Direct recovery from field	2007	Laboratory rearing 1 year	Possible descendants of KARI releases
Mombasa	Kenya	Field caught adults	2007	None	Caught on sticky traps

[1]Ethanol-preserved samples, only used for molecular studies. The size of the original field-recovered population of the CiMMYT samples was about 200 beetles. The original size number of traps used and sampling duration for the recovery of KARI and Benin populations are not known.

Figure 2.1: Map of Central America showing the field sources of *Teretrius nigrescens* used in this study. The Kr and samples from Costa Rica had been released in Africa in the 1990s founding (KARI, Benin, Ghana and Malawi populations). Population abbreviations: (Kr - KARI, Ts - Tlaltizaspan, Ox – Oaxaca, Te –Teupasenti.

2.2 Monitoring and recovery of *P. truncatus* and *T. nigrescens*

Pheromone-baited traps were used to monitor and recover *P. truncatus* and *T. nigrescens* samples in Kenya and Honduras. Disposable sticky traps (Pherocone III Delta traps, Tréce, Inc.) were used for monitoring of the flight activity of *P. truncatus*. This trap consists of a folded card loaded, sticky on the inner surface. The traps were baited with Pherocon Lures, a moisture of the two components of the LGB aggregation pheromones *Trun-call1* (1-Methylethyl (2E)-2-methyl-2-pentenoate and Trun-call 2 (1 methylethyl (2E,4E)-2,4-dimethyl-,4-heptadienoate). Delta traps were replaced every three weeks.

For live recoveries of *T. nigrescens* and LGB in Kenya and Honduras, a modification of the Japanese beetle funnel trap was used. This traps consisted of a bucket receptacle, funnel and flat circular top. The roof had a holder into which the pheromone vial was inserted and secured. And the three parts fitted into the bucket. The trap bucket was loaded with 2 grams of LGB frass and 5 grams of maize grains. The frass and maize grains had been sterilised in an oven at 40 °C for four hours (Jembere *et al*., 1995). These traps were left *in situ* throughout the survey and recovery period and serviced every fortnight. Servicing funnel traps involved removal of bucket contents for laboratory analysis and replacement of both the pheromone lure and the frass-and-maize bait.

2.3 Outgroup species

Other Histeridae species were directly sampled from the field. *Teretrius americanus* LeConte (= *T. latebricola* Lewis) was collected from bark of fallen trees around Wisconsin by Jeffrey Grubber, Russell Labs, Madison (USA). Saprophilic histerid samples were collected around Nairobi from decomposing cadavers of a cat and bird between the third and seventh days *post mortem* as well as from pitfall traps baited with liver. Histerids were also sampled from the soil underneath herbivore dung patches, compost heaps and using a flight intercept trap set near fallen tree trunks at the Kiboko Range (Figure 2.2). Outgroups species selected for this study were: *T. americanus*, *Acritus nigricornis* (Hoffmann), *Chaetabraeus* sp. (*Mazureus*), *Saprinus bicoloroides* Dahlgren, *Saprinus splendens* (Paykull), *Atholus confinis* (Erichson) and *Carcinops pumilio* (Erichson). These species were selected on the basis of their phylogenetic relationship with *T. nigrescens* (Caterino and Vogler, 2004) and sampled according to methods used by Dégallier and Gomy (1983). All insect identities were determined by Dr. Peierpaolo Vienna, Italy. Insect samples for DNA extraction were transferred to absolute ethanol and stored at -20 °C till ready for use.

2.4 Insect Rearing

Insect cultures were set up in one-litre glass jars containing maize grains. Untreated maize grains (unidentified variety) was purchased locally, cleaned and winnowed before use. A sample of every batch of new maize was exposed to adult *Sitophulis zeamais* Motchulsky (Coleoptera: Curculionidae) overnight to infer the possibility of it having been treated with insecticidal compounds. The maize was then sterilised in an oven at 40 °C for 4 hours. Maize used for laboratory tests was treated at 40 °C for four hours, as both higher temperatures and longer treatment times had been found to influence the chemical and food quality properties of the maize and subsequent insect behaviour (Jembere *et al*., 1995). Maize was allowed to cool on the bench for 24 hours at room conditions. The next day, 200 adult LGB were transferred from a healthy colony and added into the jar. This set up was either maintained at room conditions (about 27 – 30 °C) for seven weeks to multiply adult LGB for other experiments or used for *T. nigrescens* colony establishment. At every harvesting, *T. nigrescens* from several jars were mixed before being redistributed to fresh jars to increase genetic admixture.

Jars used for *T. nigrescens* rearing were kept at room conditions for one week, for LGB brood establishment, after which 20 adult *T. nigrescens* individuals were transferred from established colonies into each jar. This set up was either left until some *T. nigrescens* were needed or selected for experiments as desired. When a uniform age was essential, *T. nigrescens* adults were allowed 48 hours oviposition time after which they were sieved out. In all cases, the jars were kept in the laboratory (or incubator if for experiments) until ready for use.

2.4.1 Recognition of life stages

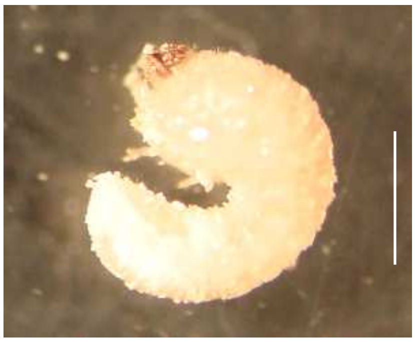
The life stages of the larger grain borer juvenile stages of were identified as previously described (Farrel and Haines, 2002) (Figure 2.3). The larval stages of *T. nigrescens* were determined though observation of development and as described by Rees (1985) and the eggs as described by Stewart-Jones *et al*. (2006) (Figure 2.4).

Figure 2.2: Flight intercept trap used to sample Histeridae predating on bark-boring Coleoptera. Insects collected in soapy water in the yellow troughs were transferred into absolute ethanol every 12 hours.

A: Adult LGB. (G. Goergen, IITA)

B: LGB egg

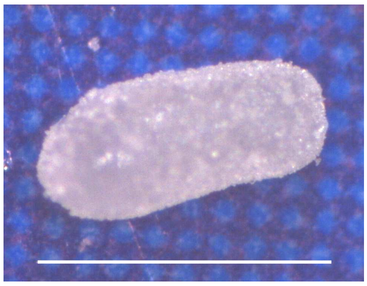
D: LGB Pupa exposed from pupal case



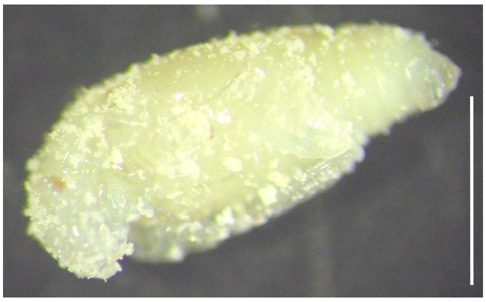
C: Third instar larva

Figure 2.3: The life stages of *Prostephanus truncatus*. The pupa was carefully removed from a pupal case for photographing. The bar in B (egg) represents 0.5 mm, for the other photographs, the bar represents 1 mm.

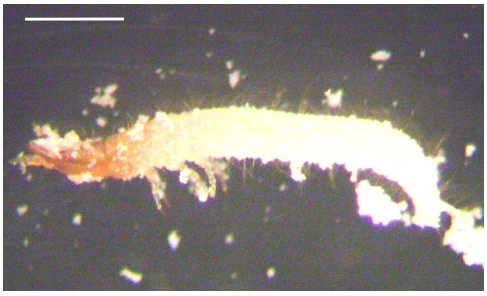
A: Freshly eclosed adult



B: Egg



D: Pupa



C: First instarLarva

Figure 2.4: The life stages of *Teretrius nigrescens.* For each photo, the bar included represents 1 mm.

2.5 General molecular procedures

2.5.1 DNA extraction

Genomic DNA

Genomic DNA was extracted from the head and thorax of individual *T. nigrescens* adults. Insect tissue was stored at -20 °C till ready for DNA extraction. For outgroup species, DNA was extracted from a single left hind leg and the rest of the specimen sent for species identification. Larger tissues of *T. nigrescens* were used to have sufficient DNA for all experimental procedures from the same samples.

The DNA was extracted using the phenol-chloroform-isoamyl alcohol protocol (Sambrook *et al*., 1989) with slight modifications. Briefly, insect tissue was homogenised in 100 µl of tissue-grinding buffer (10 mM Tris-Cl, 10 mM EDTA, 150 mM Sucrose, 60 mM NaCl, 0.5 % SDS, 25 U/ml Proteinase; pH 7.5) and incubated at 55 °C for one hour. An equal volume of the lysis buffer (0.3 M Tris-Cl, 0.1 M EDTA, 0.15 M Sucrose, 60 mM NaCl, 0.75 % SDS, pH 7.5) was added to the homogenate, mixed gently and incubated on ice for 10 min. One volume of equilibrated phenol was added to the lysate, mixed by gentle inversion and centrifuged at 5000 g for 10 min. An equal volume (200 µl) of chloroform isoamyl alcohol (24:1) was added to the supernatant in a fresh tube, mixed gently and centrifuged at 13000 g for 5 min. The DNA was precipitated by mixing the supernatant with a tenth volume of 5M sodium acetate (pH 5.2) followed by an equal volume of cold isopropanol and incubating it overnight at -20 °C. DNA was pelleted by centrifuging at 13000 g for 25 min and tilting off the alcohol gently. The pellet was washed twice with 70 % ethanol, each time centrifuging at 13000 g for 5 min before decanting off the liquid phase. It was dried in a flow hood and resuspended in 300 µl of TE Buffer (10 mM Tris pH 8.0, 1 mM EDTA). Extracted DNA was stored at -20 °C until ready for use.

Purification of PCR products

DNA was recovered from PCR products either from agarose gels or reacted PCR mix using Qiagen MinElute Gel Purification and PCR purification Kits respectively (QUIAGEN). Target DNA bands were excised from agarose gels, weighed and dissolved in to a 3 volumes of the gel solubilisation buffer in a water bath at 50 °C for 10 min. DNA was then precipitated with one volume of isopropanol and applied to a DNA-binding column. The column was centrifuged to bind to the DNA to the silica matrix while the solubilisation buffer was discarded. The column was washed once with the gel solubilisation buffer to remove traces of agarose, then washed once with the columns washing buffer (containing 70 % ethanol). DNA was eluted in 10 μl elution buffer (2 mM Tris, pH 8).

PCR products were cleaned by mixing them with five parts binding buffer (containing isopropanol) and applying to the column as for PCR gel purification above. Columns were washed with the column washing buffer and DNA eluted as already described above.

Plasmid DNA

Plasmid DNA was extracted from overnight LB colonies using the Quiprep Spin Miniprep Kit (QUIAGEN). Briefly, 3 ml of the overnight culture was centrifuged at 5000 g to pellet the bacterial cell bodies. The pellet was resuspended in a resuspension buffer with 100 μg/ml RNAse A for 5 minutes. A lysis buffer was added mixed gently and incubated at room temperature for 5 minutes. The denaturing buffer was then added to the lysis mix, quickly mixed thoroughly and centrifuged at 9000 g to separate cell debris from the lysate. The lysate was applied to a column and washed once with the binding buffer and column washing buffer respectively. Plasmid DNA was eluted in 55 µl of 2 mM Tris-Cl buffer (pH 8.0).

2.5.2 Molecular cloning

Cloning was done for eventual sequencing using pGEMT-Easy plasmid vector according to manufacturer's protocol. This plasmid vector subclones small direct PCR products generated by polymerases with A-tailing activity, without enzyme digestion. Ligation was done overnight at 4 °C. To transform competent cells, 2 µl of the ligation mix was mixed with 50 µl of freshly thawed DH5α competent cells and left on ice for 30 min. The mix was then heat shocked at 42 °C in a water bath for 50 s and immediately placed back on ice for two minutes. This mixture was added to 950 µl of SOC Medium (Promega, 2005) and grown at 37 °C with vigorous shaking for two hours. The culture was then centrifuged at 3000 g and the bacterial pellet gently resuspended in 100 µl of SOC medium. This suspension was inoculated onto Luria-Bertani/Agar plates (containing 70 mM ampicillin, 80 µg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and 0.5 mM isopropyl ß-D-thiogalactopyranoside (IPTG)) overnight for blue-white colony screening. Positive colonies were screened by PCR using the same conditions for amplifying the insert (Chapters 5 and 6).

Recombinant colonies that showed a single PCR product of definite size were grown singly in 5 ml of Luria-Bertani medium with 50 µg/ml ampicillin overnight at 37 °C with vigorous shaking to achieve high cell growth. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit as described above.

2.5.3 DNA sequencing reactions

All DNA fragments were sequenced under the BigDye terminator cycle sequencing chemistry at a commercial facility (Macrogen Inc, Korea). Products of PCR were sequenced using the same primers as those used for the respective reactions, while inserts in pGEMT-E were sequenced using universal (M13 pUC) forward and reverse primers. Ethanol-precipitated sequencing PCR products were run on an ABI 3730xl DNA Analyzer (Applied Biosystems). Where necessary, sequences were edited to remove vector sequences using VEC SCREEN Software (NCBI) and by manual alignment and editing.

2.5.4 Sequence Analysis

Apart from some very short microsatellite markers, most fragments were sequenced in both directions. Forward and reverse sequences were contigged using BIOEDIT. A local alignment of the forward sequence with the reverse complement of the reverse sequence was done around the overlapping termini (allowing two ends to slide). Once a perfect alignment was obtained, a consensus sequence was created. Ambiguous nucleotides were confirmed by comparison with the sequencing trace files. Sequence termini were then trimmed to include only portions with high chromatographic quality.

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CHAPTER THREE

The Flight Activity of *Prostephanus truncatus* (Horn) and *Teretrius nigrescens* Lewis in Kenya

3.1 Abstract

A baseline survey of the pest and its introduced natural enemy was carried out at five locations along an east-westerly transect across Kenya. The LGB was detected in all regions sampled, with higher flight activity in high potential maize growing regions in western Kenya. Its flight activity was auto correlated with the previous level and closely associated with relative humidity levels, temperature and vapour pressure deficit. There was a delayed response to meteorological variables. *Teretrius nigrescens* was only recovered after an intensive monitoring and recovery activity, in the original release region in eastern Kenya. A linear model explained 27 % of the flight activity observed and was well applicable to data collected 14 years earlier. The effect of *T. nigrescens* in semi-arid areas seems seriously affected by environmental fragmentation. We suggest an ecologically and genetically aggressive effort to ensure the establishment of the predator in Western Kenya.

3.2 Introduction

The larger grain borer spread into Kenya from Tanzania and initially established in the drier coastal and eastern regions (Hodges *et al*., 1996). Containment efforts were imposed on the movement of maize within Kenya to prevent *P. truncatus* spreading to the main maize-surplus areas in the west of the country (Hodges *et al*., 1996). Although first reported in semi-arid maize deficit areas receiving food aid (Giles *et al*., 1996), the pest has spread to the high potential production areas like the western and Rift Valley provinces in Kenya (Anonymous, 2003). This expansion poses the threats of both direct damage of bulk-stored grain and rapid dispersal from these maize-surplus regions during grain movement. At the farm-level, greater grain loss compromises the food and economic security situation in the region. The expanded range of distribution of LGB poses a new challenge to the grain industry in Kenya and the contribution of agriculture to the country's GDP.

Biological control of the LGB using *T. nigrescens* has been only partially successful in Kenya. A Mexican population was released in Wundanyi and Makueni, resulting in an 80 % reduction in pest flight activity and predator dispersal of over 70km within the first three years of release (Nang'ayo, 1996; Hill *et al*., 2003). However, the predator did not disperse beyond the semi-arid eastern areas of Kenya. Conversely, in West Africa, the release a Costa Rican population of the predator resulted in lower LGB flight activity and a reduction of damage trends was in warm humid coastal areas of Togo and Benin (Schneider *et al*., 2004). However, the predator was not efficient in the dry-hot areas. Likewise, the Costa Rican population failed to establish under the cool highland conditions of Guinea Conakry.

The work of Nansen *et al*. (2001) and Hodges *et al*. (2003) demonstrated that climatic factors could cause strong year-to-year fluctuations in *P. truncatus* flight activity even in areas where *T. nigrescens* has been present for some time. Similar fluctuations were observed for the maize weevils *Sitophilus zeamais* Motschulsky, which should not be affected by *T. nigrescens*. Hodges *et al*. (2003) designed models of LGB flight activity, with main meteorological predictors, but incorporating specific modifiers coefficients under different environmental conditions. They suggested that their models could be used to separate the effects of *T. nigrescens* from those of climate, with differences in predicted and observed trap catches of *P. truncatus* in a locality after predator introduction providing a measure of the predator's impact.

This study formed a baseline survey of the prevalence and distribution LGB and *T. nigrescens* in Kenya. It is part of a pre-release study, forming a basis of the new effort to the use of potential geographically distinct populations of the predator in the control of the LGB in all outbreak regions in Kenya. The objectives of this study therefore were to monitor the flight activity of LGB in five regions in Kenya, to model possible ecological factors influencing their abundance and to establish whether *T. nigrescens* ever spread beyond the original release area in eastern Kenya. In an attempt to separate the effect of climate factors from those of the predator the model was validated with previous work done at the Kenya site where *T. nigrescens* was first released (Hill *et al*., 2003).

3.3 Materials and methods

3.3.1 Sampling sites

Twenty trapping sites were established four each at Kakamega, Kitale, Thika, Kitui and Mombasa, which represent different agro-ecological zones (Figure 3.1). At every sampling location, traps were located at least a kilometre away from each other, and placed at least 100 m from any grain stores. Kitale and Kakamega are located in western Kenya and the Rift Valley and are characterized by cool humid conditions (Table 3.1). The latter are major maize production areas, with a tropical rainforest vegetation type. Thika is on the periphery of the semi-arid area, while Kitui and Kibwezi lie in the semi-arid lower mid-altitudes, within an early outbreak zone (Hodges *et al*., 1996). Mombasa is in the coastal lowlands, with a warm sub-humid climate, also within the first outbreak zone of LGB. Another 3-month survey was carried out in 2007 in Kibwezi and Kiboko close to Makueni to confirm the recovery of *T. nigrescens* and to provide samples for a related molecular genetics study. Kibwezi is a semi-arid area with a bimodal rainfall distribution with long rains from March to May and short rains from November and January. The land use pattern consists of scattered small-holder farmlands maize, beans and pigeon pea as the main crops. Vegetation is of the savannah woodland type characterised by diverse species of woody trees dominated by trees of the genera *Commiphora* (Burseraceae) and *Acacia* (Mimosaceae).

3.3.2 Insect trapping

Two types of traps were used. Pherocon III delta traps (Trécé Inc., Aldair, OK, USA) were used during the 28 - month survey, when the objective was to monitor insect populations. Delta traps were composed of a folded card to create a flat-based triangular shaped chamber with sticky internal sides. They were baited with the synthetic LGB aggregation pheromone mixture *trunc-call* 1 and *trunc-call* 2 (Trécé Inc.). As both *T. nigrescens* and *P. truncatus* are attracted to this pheromone, the same traps were used for both species. Traps were collected every three weeks, replaced with fresh traps and sent to the laboratory for trap quantification and identification. In total, there were 38 collections during the 28-month monitoring period from July 2004 to September 2006. Japanese Beetle Funnel traps (Trécé Inc.) were used in the three-month (February – May 2007) *T. nigrescens* survey in Kibwezi, with the aim of recovering any surviving predators alive. The traps were baited with a synthetic LGB aggregation pheromone mixture and also 50 g of sterilised maize grains and a spoonful of sterilised LGB frass (about 2 g) to arrest any arriving LGB and *T. nigrescens*. Frass and maize had been sterilised in an oven at 40 °C for four hours or 50 °C for two hours. Every fortnight, frass and maize from funnel traps were collected into labelled falcon tubes and moved to the laboratory for grain dissection and counts of adult LGB and *T. nigrescens*. This was done within a week of collection to avoid confounding results with any F1-generation that could emerge from infested grains. The target species, *P. truncatus* and *T. nigrescens*, were identified under a stereomicroscope at x10 magnification and counted.

Table 3.1: The geographic and climatic conditions in the five monitoring regions in Kenya

Region	Altitude (m a.s.l.)	Rainfall (mm)	TMax (°C)	Tmin (°C)
Mombasa	0 - 500	1200	29.4	20.0
Thika	500 -1200	500 - 1000	28.6	16.4
Kitale	1000 - 1500	800 - 2500	23.0	10.0
Kakamega	1000 - 1500	1000 - 2000	23.3	13.4
Kitui	200 - 400	600	32.0	22.0
Kibwezi	100 - 500	600	31.0	20.0

Tmin and TMax = average annual minimum and maximum temperatures respectively.

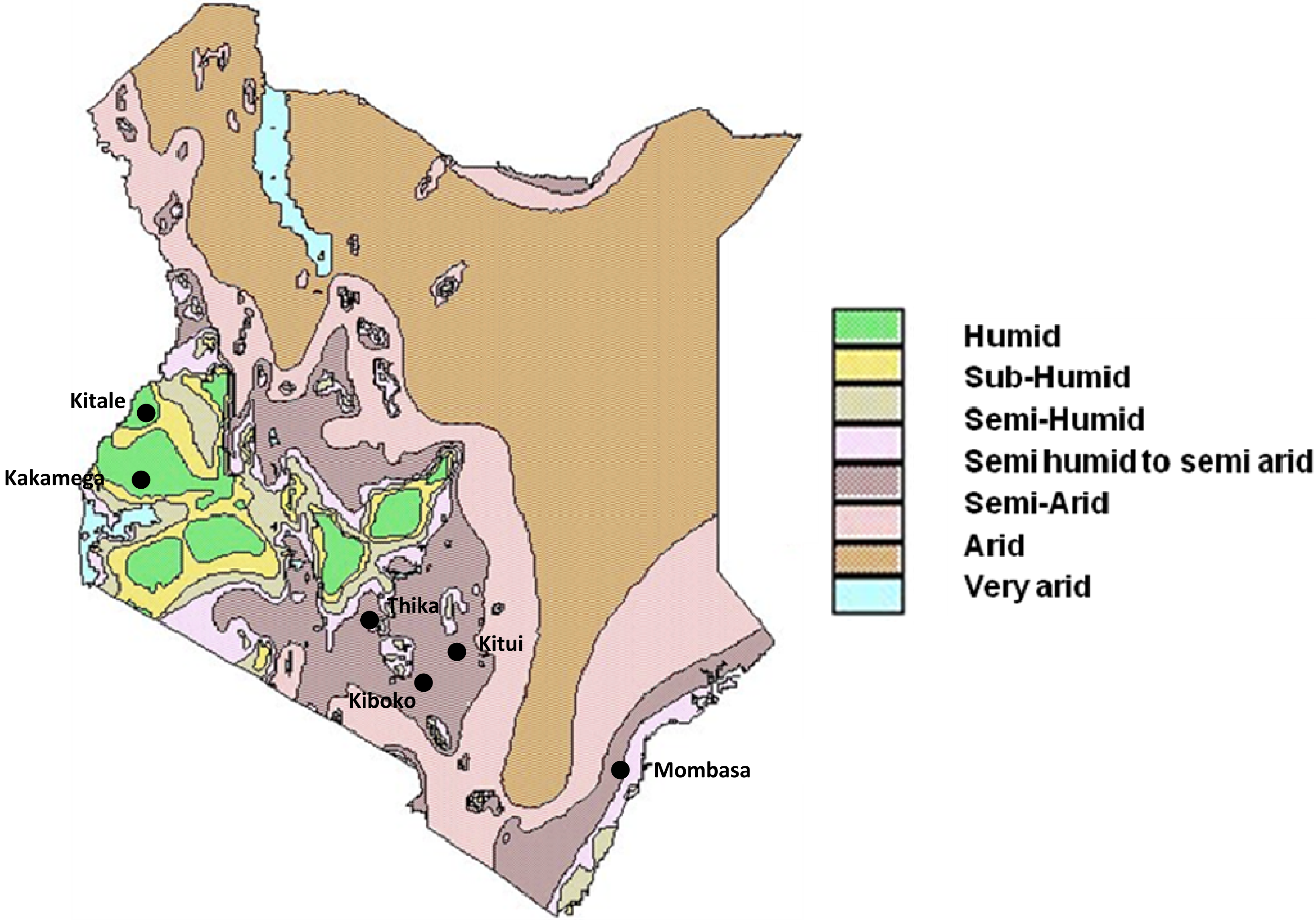

Figure 3.1: Map of Kenya showing major agro-ecological zones and the locations of sampling stations monitored for LGB and *T. nigrescens* flight activity on Kenya between 2004 and 2007 (Source: USDA, 2004: http://www.fas.usda.gov/pecad/highlights/2004/12/Kenya/images)

3.3.3 Meteorological data

Weather stations were present at all trapping sites except for Kitui and Kibwezi, for which data from the nearest site of Makindu was used. Monthly rainfall, minimum (Tmin) and maximum temperature (TMax), relative humidity (RH_6, RH_{12}) and dew point at 0600 and 1200 hours (DP_6, DP_{12}, respectively) were obtained from the Kenya Meteorological Department. Relative humidity and dew point data were used to calculate monthly vapour pressure deficit (VPD).

3.3.4 Data analysis

Because LGB data was collected about every three weeks, the average monthly climatic data was converted to the same time scale using the interpolation function of MATLAB (MATLAB, 2000) to enable the assessment of the relationship between LGB trap catches and climatic variables. These data were then analysed by means of classical principal component analysis (PCA) on covariance matrix (Devillers and Karcher, 1991). Briefly, PCA replaces the original variables of a data set with a smaller number of uncorrelated variables called principal components (PCs). The method is linear in that the new variables are a linear combination of the original ones. The first principal component (PC1) accounts for the most part of the variance of the system, followed by PC2, PC3, and so on. PCA was performed on data converted into their log10 value.

The study of the relationships between the LGB and the environmental variables were investigated by means of partial least squares (PLS) regression analysis (SAS, proc PLS). The climatic variables and number of LGB are centred by subtracting their means and scaled by dividing by their standard deviations.

Standardised x: $x = \frac{x_i - \bar{x}}{s}$

LGB data from each site at Mombasa, Kakamega, Kitale and Thika at time *t* were regressed using the partial least squares (PLS) model with climatic data as predictors and with or without LGB as auto-correlated factor. The order of the LGB autocorrelation model was determined by an autoregressive moving average (ARMA) models (proc ARIMA, SAS). The PLS procedure was used to fit models when the predictors were highly correlated, which is possible for climatic variables, with LGB as an independent factor. It sought to determine the minimum number of uncorrelated factors that significantly explain both LGB flight and climatic variations. The model was validated to the LGB data from a survey done at Kibwezi between 1991 and1997 (Hill *et al*., 2003).

3.4 Results

3.4.1 <u>Seasonal fluctuation in numbers of LGB and *T. nigrescens*</u>

The highest mean trap catches were recorded in Kakamega followed by Kitale. They were considerably lower and did not vary significantly in the other three locations (Figure 3.2). Average trap catches varied from 30 per trap in Mombasa to 550 in Kakamega, with a peak of over 2000 in July, 2005. LGB flight activity was bimodal in Thika and Kitui, with a major peak in November-January and a minor peak around March – May, just following the short rain season and at the beginning of long rain season.

No *T. nigrescens* was trapped during the 28-month monitoring period in these five locations. During the intense survey in Kibwezi, only three adults of *T. nigrescens* were caught in the Kiboko range and none at the original release site in Kibwezi area. Four adults were caught in a single sentinel 2-week trapping period at one site in Mombasa.

Projection of the LGB flight into its principle components did not lead to a separation of the different sites and the models were therefore applied to all four regions. The first two principal components 1 and 2 contributed 58.02 % and 25.60 % to the total variance, the third, to the seventh components contributing 16.39 % (Table 3.2). Dew point, minimum temperature and rainfall contributed positively to both principle components.

Table 3.2: Eigenvalues and weights for the first two principal components computed from $\log_{10}$ transformed meteorological data.

Variable	Weight PC1	Weight PC2
Proportion of covariance	0.5802	0.2560
Eigenvalues	0.0412	0.0219
Maximum Temperature	-0.371	0.441
Minimum temperature	0.049	0.669
Relative humidity at 0600 hrs	0.431	-0.259
Relative humidity at 1200 hrs	0.456	-0.157
Dew point 0600 hrs	0.403	0.371
Dew point 1200 hrs	0.382	0.350
Rainfall	0.397	0.077

Table 3.3: Percent variation accounted for by partial least squares factors

Factors[1]	Model effects		Dependent variables	
	Current	Total	Current	Total
1	51.675	51.675	26.152	26.152
2	24.706	76.381	8.037	34.189
3	9.097	85.478	4.021	38.211
4	3.058	88.536	1.091	39.302
5	3.959	92.495	0.226	39.528

[1]Number of variables included into the variable building

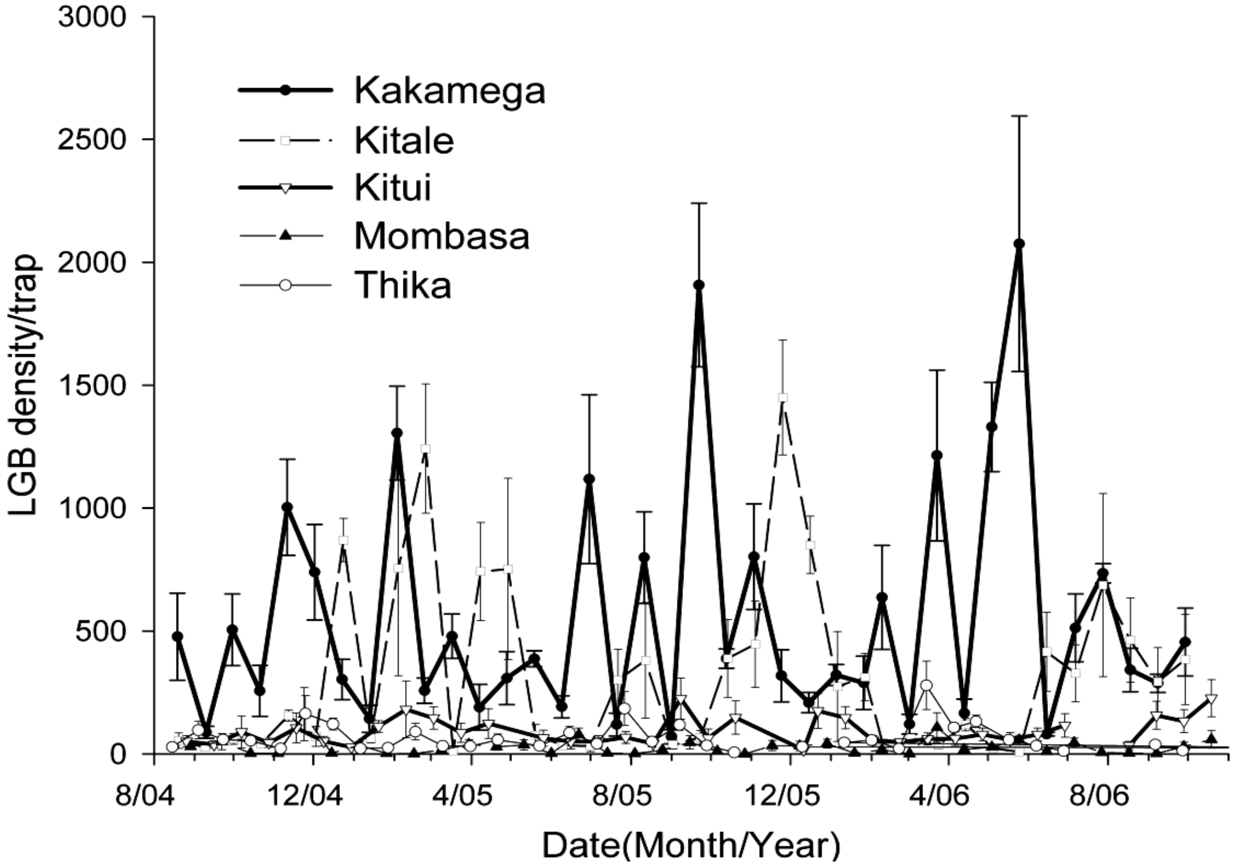

Figure 3.2: The flight activity of *Prostephanus truncatus* in five regions in Kenya (2004 – 2007).

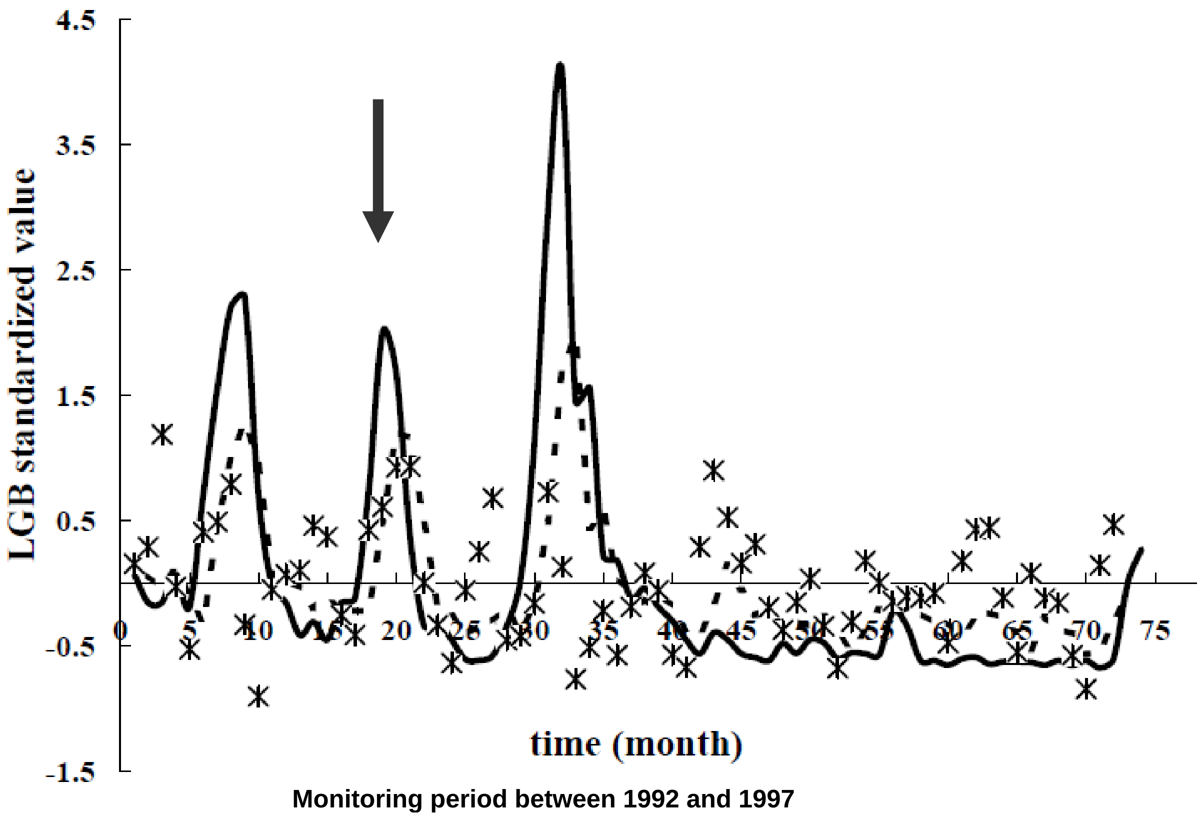

Figure 3.3: The temporal dynamics of standardized value of observed number of Larger Grain Borer (solid line), predicted LGB with LGB as auto-correlated factor (dash line), and predicted LGB without LGB as auto-correlated factor (star) in Makueni between 1991-1997. X-axis represents sampling times with the unit of time (month), and y-axis is the standardized value of LGB by the deviation with its mean value divided by the standard deviation. Arrow indicates time of first *Teretrius nigrescens* release in Makueni in 1992.

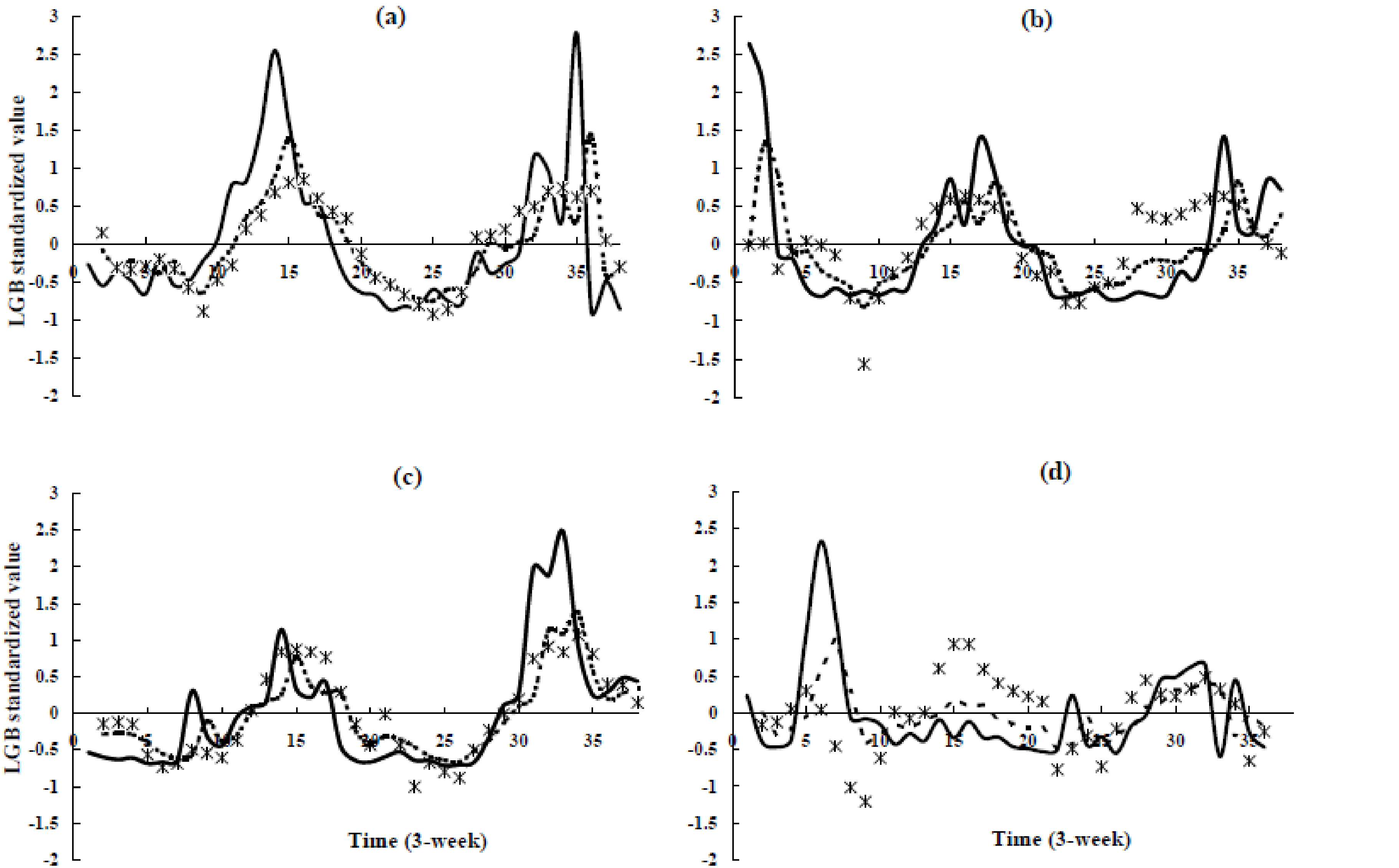

Figure 3.4: Figure 3. The temporal dynamics of standardized value of observed number of larger grain borer (solid line), predicted LGB with LGB as auto-correlated factor (dash line), and predicted LGB without LGB as auto-correlated factor (star) at 4 sites: (a) Kakamega; (b) Kitale; (c) Mombasa; (d) Thika. X-axis represents 38 sampling times between 2004-2006 with the unit of time (3-week), and y-axis is the standardized value of LGB, which is the deviation with its mean value divided by the standard deviation.

Table 3.4: Parameter estimates for seven variables used to predict the flight activity of the LGB in four zones in Kenya.

Source/Parameter	**Type I SS**		**Type III SS**		**t value**	**P > |t|**
	F	**P**	**F**	**P**		
Maximum Temperature	130.33	0.000	10.16	0.002	-3.19	0.002
Minimum temperature	0.26	0.608	0.12	0.730	0.35	0.730
Relative humidity at 0600 hrs	23.27	0.000	28.13	0.000	5.30	0.000
Relative humidity at 1200 hrs	19.82	0.000	11.74	0.001	-3.43	0.001
Dew point 0600 hrs	0.22	0.638	0.02	0.886	0.14	0.886
Dew point 1200 hrs	0.15	0.700	0.16	0.693	-0.40	0.693
Vapour Pressure Deficit	0.47	0.495	0.47	0.495	-0.68	0.495

Table 3.5: Parameter estimates for data percentage contribution to model estimates.

	Centred	Scaled
Intercept	0.000	-0.014
LGB(*t*)	0.383	0.377
Maximum Temperature	-0.231	-0.224
Minimum temperature	0.126	0.123
Relative humidity at 0600 hrs	0.386	0.374
Relative humidity at 1200 hrs	-0.256	-0.247
Dew point 0600 hrs	0.012	0.012
Dew point 1200 hrs	-0.083	-0.080
Vapour Pressure Deficit	-0.034	-0.591
Rainfall	-0.051	-0.050

3.4.2 The LGB flight model

1) The model without LGB number as an auto-correlated variable

From the CV test of cross-correlation analysis of the PLS model, the minimum number of 6 variables, contributed 96 % of the variation in the predictors but only explained 22 % of the variation in responses ($R^2 = 0.281$, $F = 24.93$, $p < 0.0001$) (Table 3.4). The following model was thus derived:

$$LGB(t) = -0.97*Tavg+0.65*TMax+0.43*Tmin+0.37*RH_6-0.32*RH_{12}-0.32*VPD$$

2) With LGB catches as an auto-correlated variable

Time series analysis indicated that LGB dynamics at all sites followed an AR (1) model, in which LGB at time *t* is dependent on time at *t* - 1. Therefore, LGB at *t* - 1 was considered as one of the factors. From the coefficient of variation test of the cross-correlation analysis of the PLS model, the minimum number of three variables significantly contributed 87 % of the variation in the predictors and 37 % of the variation in responses (Table 3.5). To determine which factors to eliminate from the analysis, the regression coefficients of each factor in the PLS model, which determines the importance of each factor in the prediction of the response, were compared. If a predictor had a relatively small coefficient (in absolute value), then it was considered a prime candidate for deletion.

The results from Kakamega, Kitale, Mombasa and Thika sites indicated that LGB at time *t* was affected by LGB at the previous sampling time (LGB[t-1]), RH_6 and TMax.

$$LGB(t) = -0.023+0.486*LGB(t-1)+0.122*RH_6-0.049*TMax$$

Figure 3.4 shows observed and predicted LGB trap catches. There was a one unit time delay due to the time series model.

In Kibwezi, LGB trap catches during the February – June 2007 period were low (< 300 beetles per fortnight) with most catches below 50 beetles per fortnight. However, traps located near markets or established grains stores yielded higher LGB numbers than those located further inland. Still, some LGB was detected in the Kiboko Range, 10km away from the nearest homestead of a maize farmer.

3.5 Discussion

In Africa, the spread of *P. truncatus* could be related to movements of contaminated maize consignments, usually from maize surplus to maize deficit areas (Bosque-Pérez *et al*., 1991; Adda *et al*., 1996; Schneider *et al*., 2004). Thus, as also reported for West Africa, in Kenya trap catches were higher close to roads and markets than in the interior where homesteads were sparse. The dispersal of LGB beyond the maize deficit areas of eastern Kenya to the high production areas in the west took more than ten years after it was first discovered in the country. It is most likely that the infestations in western Kenya stemmed from maize importations from Tanzania during lean years into deficit areas close to Kitale and Kakamega (Anonymous, 2003)

Though LGB reached western Kenya in recent years only, trap catches were considerably higher in these high production areas than in the maize deficit areas of eastern Kenya, where the pest was introduced in the country in the early 1990s (Hodges *et al*., 1996; Giles *et al*., 1996). They were comparable to those in maize surplus areas of Ghana and Benin prior to the introduction of *T. nigrescens* (Schneider *et al*., 2004). This appears to be indicative of an introduction of the pest into the region without *T. nigrescens* or the inability of the predator to establish as a result of climatic limitations. However, according to the regression model of Tigar *et al*. (1994), who used average relative humidity, annual rainfall and annual mean temperature as independent variables, maximum catches in pheromone traps are expected at temperatures of 23 – 25 °C and relative humidity of 50 – 52 %. This would suggest high trap catches in the cool areas of western East Africa corroborating the results of the present study.

In Kitui, Kiboko and Mombasa, LGB trap catches were considerably lower than in western Kenya. Kitui and Kiboko lie in a maize deficit hot-dry zone with sparse shrub land but with several alternate tree hosts of LGB (Nang'ayo, 1996). The sustained trap catches of below 200 adults per trap per month were significantly lower than those observed in the same region before the release of *T. nigrescens* and comparable to the flight activity recorded after the establishment of the predator in the area (Giles *et al*., 1996; Hill *et al.*, 2003). However, they were in the range of those in the Guinea and Sudan Savannas in West Africa, where *T. nigrescens* appeared not to exert control over LGB (Schneider *et al.*, 2004). Not a single adult *T. nigrescens* was captured during the two year survey in the mid-altitudes, and just three specimens in the more intensive survey in the Kiboko range, where no single trap captured the predator more than once during three-month trapping period using 51 traps. In the coastal area around Mombasa, trap catches were even lower than in the semi-arid mid-altitudes. However, the traps were located in the south coast where both precipitation and maize production is low compared to the hot-humid coastal area of West Africa, where *T. nigrescens* appeared to control *P. truncatus* (Schneider *et al*., 2004). In the coastal area only four *T. nigrescens* specimens were captured. Hill *et al.* (2003) recovered an average of one *T. nigrescens* adult in every 20th trap in early 1995 and none during a three-year monitoring period thereafter. By contrast in Benin, Borgemeister *et al*. (1997a; 2001) recorded 100 – 400 *T. nigrescens* per trap per month, while Schneider *et al.* (2004) observed 20 – 110 adults per trap in the forest savannah region, and about two insects per trap in the drier northern Guinea savannah. Though the presence of predator in eastern Kenya confirms its establishment and spread over 15 years since its release, the very low numbers recovered suggest that the predator is not responsible for the low LGB trap catches.

The introduction of a natural enemy results in an initial increase in the population of the natural enemy and a concomitant decrease in the number of the pest species, followed by a reduction in both species as they, ideally, level off into stable equilibrium (Hoddle, 2001). In Kenya, although this trend was initially observed during the first few years after releasing *T. nigrescens* in Kibwezi (Hill *et al.*, 2003), the predator is now exceedingly rare, and in some areas LGB catches have gone back to almost the levels before the release, e.g. high catches of up to 200 individuals per trap were observed close to the Kiboko Range station during the second intensive survey. The question arises if the original decrease in pest densities were due to *T. nigrescens* and why the predator so scarce in the area.

According to Zabel and Tscharntke (1998), the effect of habitat fragmentation depends on the food specialization of the species, whereby monophagous herbivores have a higher probability of being absent from small patches than polyphagous species. In addition, species richness of herbivores was shown to be positively correlated with habitat area while species richness of predators appeared to be negatively correlated with habitat isolation. They argued that due to instability of higher trophic level populations and limitations to disperse, predators were more affected by habitat isolation than herbivores. Nansen *et al*. (2002) and Hill *et al.* (2003) showed that the flight activity of *P. truncatus* was consistently higher near maize stores than inside forests, underlining the superior quality or attractiveness of maize compared to woody substrates, which was demonstrated by Hill *et al*. (2003). The original release area consists of scattered smallholder farmland, where crop fields are surrounded by savannah woodland, thus maize fields and stores are relatively scarce. Furthermore, because of insufficient rain, grain deficits are common in the area because maize harvests fail in 50% of the seasons (Anonymous, 1983). Thus, the relative scarcity of stored grain, when compared to the high-production zones in western Kenya, the low maize production potential and the frequent crop failure should lead to a highly fragmented habitat in time and space. In addition, LGB is an oligophagous species mainly feeding on stored products that allow for much higher population growth rates than woody substrates (Hill *et al*., 2003), whereas *T. nigrescens* is highly monophagous (Rees *et al*., 1990; Scholz *et al*., 1998). Thus according to Pimm (1991), Lawton (1995), Holt (1996) and Zabel and Tscharntke (1998), the predator should be more susceptible and go extinct more readily than its phytophagous prey if habitat fragmentation increases. It is hypothesized therefore that in eastern Kenya, due to habitat isolation and lack of habitat connectivity as a result of the low number of release sites, *T. nigrescens* went locally extinct. It would also follow that in the semi-arid and humid coastal areas of Kenya, the role of wild, woody host plants in maintaining LGB and predator populations as suggested by Nang'ayo (1996), Borgemeister *et al.* (1998a,b) and Nansen *et al*. (2002) is limited. It is recommended to release it again, but with a much higher number of release sites and released populations to increase the interconnectedness between the different populations occupying cultivated and wild habitats. It is also suggested that the chances of permanent establishment of *T. nigrescens* are higher in the high production, maize surplus areas in western Kenya than the maize deficit areas in the eastern semi-dry mid-altitudes given the predator strain is adapted to the cooler climates.

The effect of weather parameters on the LGB abundance and flight activity was not clear cut. Auto-regression of LGB appeared to be more important than weather factors in explaining seasonal variation in LGB trap catches. Another reason responsible for the lower significance of the weather variables is that at each site, the same meteorological parameters were applied to four different traps, which could not reflect the real micro-changes in climate. This study agrees with Hodges *et al*. (2003) who observed that flight activity is affected by weather parameters, mostly in their effects on the available population of LGB adults and their influence on triggering flight. Earlier studies had identified temperature, rainfall and humidity as the major abiotic determinants of LGB flight activity in Central America, West and East

Africa (Tigar *et al*., 1994; Nang'ayo, 1996; Nansen *et al*., 2001; Hodges *et al*., 2003). However, other studies suggest that sharp rises in flight activity at onset of rains is caused by the response to environmental cues rather than rapid reproduction leading to overcrowding and substrate degradation that may also trigger dispersal (Fadamiro and Wyatt, 1995; Hill *et al*., 2002). Borgemeister *et al*. (1997b), on the other hand, speculated that peak trap catches were related to storage patterns, coinciding with cleaning of grain stores for restocking. By contrast, Nang'ayo (1996) observed a significant correlation between the onset of flight with RH and temperature and found that flight peaks were not associated with maize storage.

It is not clear whether the decrease in LGB trap catches observed in the 90s in the release area were due to the predator or due to a combination of climatic factors which also affected yields and therefore amount of stored maize. In Ghana, Hodges *et al*. (2003) could attribute seasonal and yearly fluctuations of LGB solely to climatic factors and there was no clear-cut decrease in trap catches after the release of the predator. Similar observations were made by Nansen *et al*. (2001) in southern and central Benin using climatic models contradicting results by Borgemeister *et al*. (1997b) and Schneider *et al*. (2004), who had however used a much higher number of pheromone traps over a wider area. Nansen *et al*. (2001) found that the same climatic parameters determined flight in various locations in southern Benin, whereas for central Benin new coefficients for the same environmental variables were needed to produce adequate predictions, while for northern Benin the model could not predict flight behaviour of LGB. In addition, different driving variable were used by Hodges *et al*. (2003) and Nansen *et al*. (2001), which indicate that other environmental variable not measured in the two studies are required to give reliable predictions of LGB.

Due to the very low number of the predators caught and the erratic nature of their recovery, it is not possible to directly evaluate their effect in this survey. Such paucity of the predator punctuated with long periods of no predator capture in flight traps had been observed by Hill *et al*. (2003), even when LGB flight activity remained low. Hill *et al.* (2003) recovered an average of a single *T. nigrescens* adult in every 20th trap in early 1995 and none during a five-year survey thereafter. In Benin, Borgemeister *et al.* (1997a; 2001) recorded a predator prey ratio of approximately 0.025 in the drier North to 0.15 in the maize surplus hot-humid regions in the South. Schneider *et al*. (2004) observed a lower sustained activity of about 20 adults per trap per month in the forest savannah region and about two insects per trap in the drier northern Guinea savannah.

Flight traps sample individuals in the dispersal phase and may not be a reflection of the actual population sizes (Hodges, 2002). The use of prey pheromones may also not be optimum for the predator species, whose attraction depends on the nutritional and physiological state too (Larsson *et al*., 2009). However trap catches over a long period may still reflect the relative abundance of the pest (Hodges, 2002; Schneider, 2004). The predator-prey ratio in the drier parts of eastern and the coastal regions of Kenya were lower than those observed in the dry regions of northern Benin. In northern Benin, peak ratios of 0.01 were observed compared to about 0.03 in Kenya in 1995 (Nang'ayo, 1996; Nansen *et al*., 2001; Hill *et al*., 2003; Schneider *et al*., 2004). In Mombasa, Kenya, predator–prey ratio was as high as 0.02 per trap during the brief survey that took just a month. In Mexico, Tigar *et al*. (1994) observed ratios of 0.01 in the maize surplus area of La Laguna where *P. truncatus* was abundant. However, Rees *et al*. (1990) observed much higher predator-prey ratios of between 0.24 and 0.48 with higher ratios in maize production areas of Mexico using fewer traps and for a one-month survey. These sharp variations in the predator-prey ratios in Central America and Africa are interesting.

The establishment and success of the predator in Kenya is lower than that in West Africa. Differences between the two release approaches exist. In West Africa, while about 130 000 were released over a six month period in Togo alone, Kenyan releases were about 4 900 insects (Giles *et al*., 1996). This single small release may have compromised the ability of the predator to find and establish in suitable habitats in suitable numbers. The strain released in West Africa was also sourced from the same latitudinal range as the release point, a factor in eventual success of the natural enemy (Phillips *et al*., 2008). This study concludes that although *T. nigrescens* may be playing a role in controlling LGB populations, it is unlikely to be a key source of LGB population regulation in Kenya at the moment. A more genetically and temporally aggressive release especially in western Kenya holds promise for the success of the biological control of the LGB. The rest of this thesis examines the ecological and genetic parameters of geographical populations of *T. nigrescens* that might be candidates to these new efforts.

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CHAPTER FOUR

Effect of temperature and humidity on the predation of *Teretrius nigrescens* on *Prostephanus truncatus*

4.1 Abstract

The variable efficacy of two geographic populations of *Teretrius nigrescens* has led to the hypothesis that ecological strains of the predator exist. Thus sustainable biological control of the larger grain borer in Africa could benefit from the exploitation of this strain diversity. To test this hypothesis, five putative strains of the predator were tested for their ability to multiply, control LGB, and to prevent maize kernel damage at 18, 21, 24, 27, 30, 33 and 36 °C and under low (40 – 55 %) and high (70 - 85 %) relative humidity. Both the LGB and *T. nigrescens* performed better under humid than dry conditions. Between 21 and 33 °C, *T. nigrescens* reduced LGB population build up by about 80 %. Although the predator could not exert as much control under extreme conditions, adult survival was high at both the lower and upper extremes

4.2 Introduction

Teretrius nigrescens was introduced into Africa, after studies showed that it was an effective predator closely coadpted with its prey's biology and habitat. *T. nigrescens* locates its prey by cuing in on its aggregation pheromones (Rees *et al.*, 1985,1990; Böye, 1988; Böye *et al.*, 1988). This enhances natural dispersal and efficacy of the predator in the wild (Nang'ayo, 1996; Schneider *et al.*, 2004). However, its establishment has been population and climate-dependent. Although a Costa Rica population established well and efficiently contributed to the control of LGB in the warm humid regions of southern Benin and Togo, it was not efficient in the dry, hot savannas (Borgemeister *et al.*, 2001; Schneider *et al.*, 2004). In Kenya, a Mexican population established in the warm semi arid regions of eastern Kenya, but flight activity has remained very low (Hill *et al*., 2003). Both populations did not establish in the cooler highland regions of Guinea Conakry and Kenya (Giles *et al*., 1996; Anonymous, 1999).

Both LGB and *T. nigrescens* have been recovered from both sub-humid and semi-arid zones across Central America (Rees *et al*., 1990; Tigar *et al*., 1994). It is therefore possible that the failure of the predator to establish in parts of Kenya could have been a result of suitability of local ecological conditions around the release sites for the strains of the predator released. The larger grain borer has now spread over most ecological environments in sub-Sahara Africa between southern Senegal and the Venda region of South Africa. Sustainable development of biological control components, therefore, involves predator populations adapted to the diverse climatic conditions in Africa. Four new populations of the predator and two previously released ones are currently being considered for release in eastern Africa. Based on the preliminary observations of genetic diversity and ecological preference of *T. nigrescens*, the suitability of each population for specific regions should be determined before release.

This study investigates the effects of fourteen constant temperature and humidity combinations on the predation of the LGB by five putative strains of the *T. nigrescens* on shelled maize. The response of *T. nigrescens* is measured in terms of population increase, while predatory performance are measured in terms of control of LGB population growth, LGB mortality, grain damage and grain weight loss.

4.3 Materials and methods

4.3.1 <u>Insects</u>

The colony of *P. truncatus* used in this experiment originated from a laboratory culture originally recovered from Kitale (Kenya) in 2004 and maintained at room temperature on maize kernels in the Animal Rearing and Quarantine Unit (ARQU), ICIPE (Chapter 2). All maize used in the experiments was purchased locally. Five putative strains of *T. nigrescens* were used in this study. Three colonies recovered from Mexico by CIMMYT – Mexico from Batan, Tlaltizaspan and Oaxaca. The Kenyan population was obtained from the Kenya Agricultural Research Institute (KARI) Kenya Kiboko Field Station. This was the remnant of a colony originating from around Nuevo Leone, Mexico, initially released for the control of the LGB in Kenya in 1992 (Nang'ayo, 1996; P. Likhayo, KARI, Kenya, R. J. Hodges, NRI, UK, personal communication). The Benin population was donated by the Centre for Biological Control, International Institute of Tropical Agriculture (IITA) in Benin. This population had previously been released in Benin and Togo in 1993. The colony was maintained in the laboratory on LGB on shelled maize with regular introgression with field collected samples, the last being in 1996 (C. Atcha, WARDA, Ivory Coast, personal Communication). In our laboratories *T. nigrescens* were reared on maize grains and LGB collected from Kitale. All insects were reared under similar conditions for at least six months before use in experiments to minimise the effects of past rearing conditions and hence have a common starting point.

4.3.2 Experimental set up

The effect of temperature and humidity conditions on the ability of *T. nigrescens* to suppress LGB populations was studied under eight temperature levels i.e. 15, 18, 21, 24 27, 30, 33 and 36 °C and two relative humidity regimes (low: 45 – 55 and high: 75 – 85 %) in environmental chambers (Sanyo, Japan). One hundred unsexed *P. truncatus* adults were introduced into 250 ml glass jars containing 100 g of hand-selected, undamaged maize kernels, sterilised at 50 °C for four hours in an oven (Jembere *et al.*, 1995). The number of grains per jar, recorded for eventual damage determination, averaged 228.4 ± 13.2. To each jar, 100 unsexed LGB adults were added and left at room temperature for a week to allow for oviposition. From each strain, twenty unsexed, adult *T. nigrescens*, less than 3 months old, were then added to each jar. The jars were closed and kept in an incubator at the respective constant temperature and humidity conditions for 70 days. Two controls, one with *P. truncatus* alone and the other with uninfested maize were also set up. Each treatment was replicated six times.

To assess grain damage, the substrate mixture was sieved with a 3.5 mm and 0.4 mm mesh sieve (Helbig, 1999). Particles passing through the 3.5 mm mesh sieve were classified as loss and those passing through the 0.4 mm mesh as insect frass. The fraction retained by 3.5 mm mesh was sorted by hand into damaged and undamaged grain and counted. The number, weight and moisture content of damaged and undamaged grains and weight and moisture content of LGB frass were determined. Damaged grains were dissected and the insects counted. Insect counts were restricted to adults, to limit the data to the first generation individuals and to avoid error from possible decomposition of dead larvae due to their soft body tissues. The moisture content of the grains in the uninfested control was used to account for grain moisture content in determining grain damage.

4.3.3 Damage and loss assessment

In this study, the terms 'grain damage' and 'grain loss' were used *sensu* Boxall (2002). Damage refers to the proportion of the grain found to bear signs of LGB attack while loss was restricted to a decline in grain weight. As LGB caused near total grain destruction, grain damage was determined by posterior subtraction of uninfested grain in each set up at the end of the experiment from the initial number of grains, i.e.

Grain damage % = 100 x [(a-b)/a]

where a was the initial number of grain and b the number of undamaged grain at the end of the experiment.

Weight loss was determined as

Weight Loss % = 100 x $\{[W_a\text{-}(W_b+W_c)]/W_a\}$

where W_a was the initial grain weight grain, W_b the weight of undamaged grains and W_c the weight of damaged grain at the end of the experiment.

LGB and *T. nigrescens* population build-up was determined as the final number of individuals counted (adults only for LGB) minus the initial number of individuals added at set-up of the experiment. Insect mortality was determined as the number of dead adults of the respective species counted as a fraction of the total number of adult insects recovered at the end of the experiment. Juvenile stages were excluded to avoid error due to the possible decomposition of their soft body tissues.

The index of grain loss prevention by *T. nigrescens* (Figures 4.4 and 4.5) was determined at each temperature level as:

Grain loss prevention index = 100 x $(1\text{-}\ (D_c - D_i)/D_c))$

Where D_i is the mean loss for each strain and D_c mean loss for untreated control.

4.3.4 Data Analysis

Two-way analysis of variance (ANOVA) was performed in SAS (SAS Institute, 2000) using the general linear model (proc glm) with temperature, humidity and *T. nigrescens* strains as the main effects. Data on insect counts (LGB and *T. nigrescens*) were $\log_{10}(x)$ and $\log_{10}(x + 1)$ transformed before ANOVA procedure in SAS. Damage level data (as a proportion of the original grain numbers and weight) were arcsine square root transformed before ANOVA.

When ANOVA showed significant differences, mean separation was done using the Student-Newman-Keul (SNK) test (in SAS). Untransformed data are presented.

4.4 Results

Teretrius nigrescens survived the ten weeks of the experiments under all temperature and humidity regimes but did not reproduce at 15 °C, thus this temperature was removed from further studies. At 18 °C, under both humidity regimes, both *T. nigrescens* and *P. truncatus* did not develop to maturity within the 10 weeks though juvenile stages of both species were observed. *Teretrius nigrescens* population growth was highest at 30 °C for all populations tested (Figure 4.1 and 4.2) apart from the Benin population that performed best at 27 °C.

The effect of *T. nigrescens* on LGB population growth is summarised in figures 4.3 and 4.4. The LGB populations increased under all temperature conditions. The effect of *T. nigrescens* in reducing LGB population growth was over 80 % between 21 – 33 °C. Above 33 °C the effect of *T. nigrescens* appeared higher than at the other temperature levels. This is however a possible combination of the effect of this temperature on both species and the exaggerated effect of minimal predation by surviving *T. nigrescens* adults.

Slight differences between the *T. nigrescens* strains' ability to control the population build up and confer grain protection against the LGB were observed (Figures 4.5 and 4.6). The KARI population appeared less efficacious than the other populations at low temperature levels, but similar under high temperature conditions. For instance, grain weight loss was significantly higher with the KARI population at 21 °C ($F = 20.9$, $p < 0.0001$) and 24 °C ($F = 38.8$, $p < 0.0001$) while it was not significantly different from the other populations at higher temperature levels (Table 4.1). Similar trends were observed for survivorship and LGB population increase. The other populations did not differ in their ability to control grain damage.

Grain damage trends were generally similar to the weight loss trends but they were more consistent (Table 4.1). The effect of humidity was generally more discernable above 24 $^{\circ}$C where higher humidity resulted in higher grain damage. At low temperature levels, the effect of humidity was minimal. There were no discernable trends in mortality levels of *T. nigrescens* and *P. truncatus* between treatments.

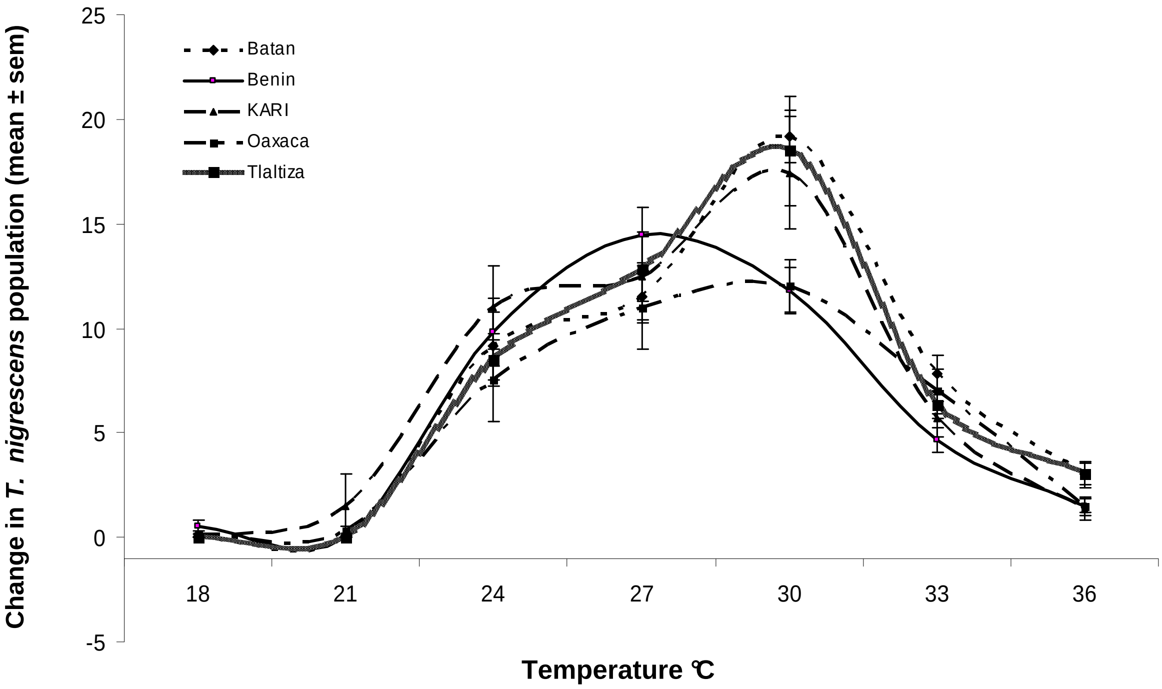

Figure 4.1: The effect of temperature on the population increase of *Teretrius nigrescens* under 40 – 55 % relative humidity regime.

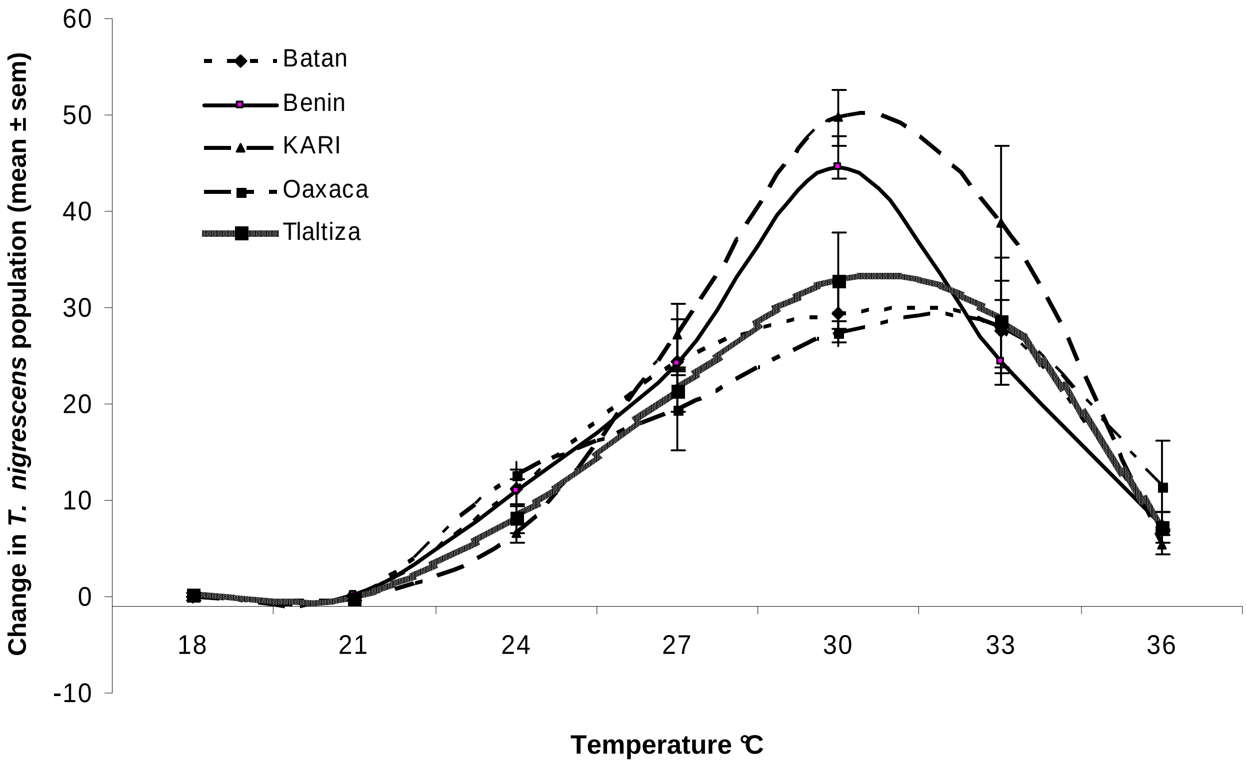

Figure 4.2: The effect of temperature on the population increase of *Teretrius nigrescens* under 40 – 55 % relative humidity regime.

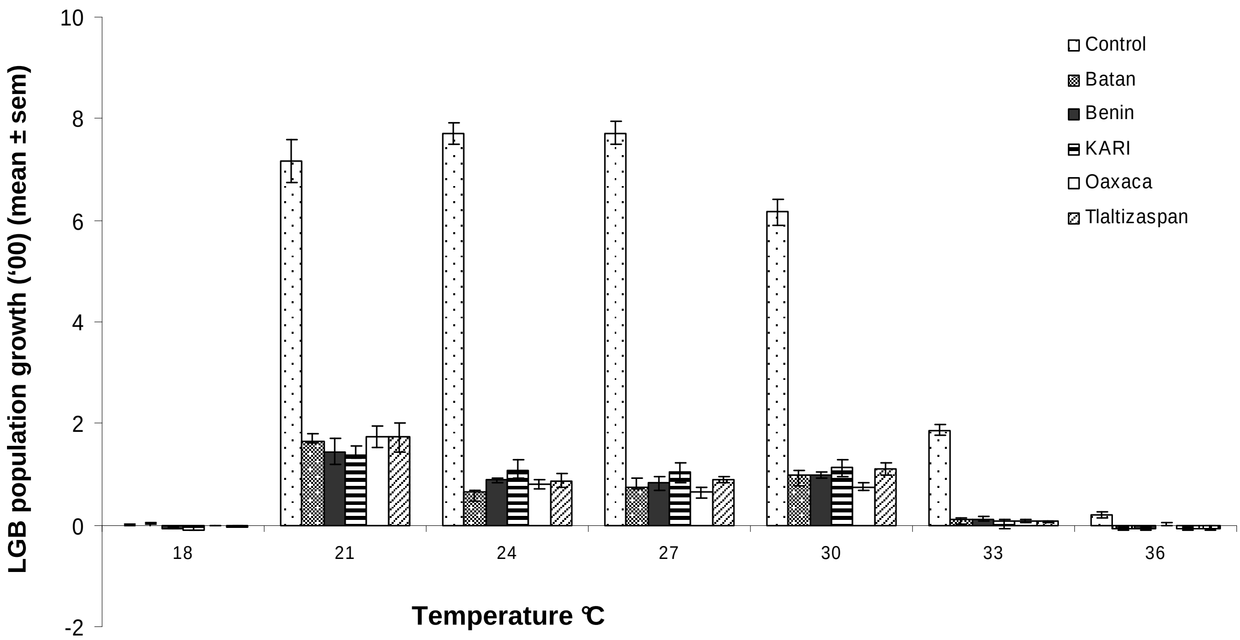

Figure 4.3: Effect of *Teretrius nigrescens* on LGB population growth at six temperature conditions at 40 – 55 % relative humidity.

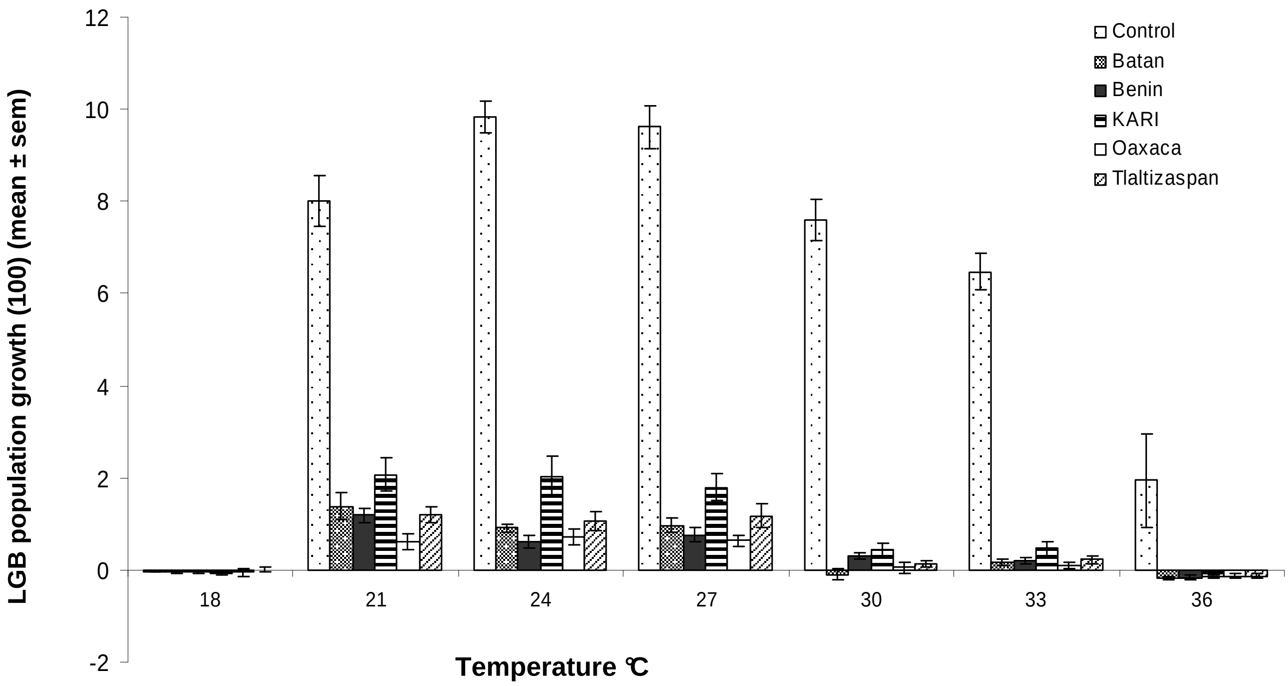

Figure 4.4: Effect of *Teretrius nigrescens* on LGB population growth at six temperature levels at 70 – 85 % relative humidity conditions.

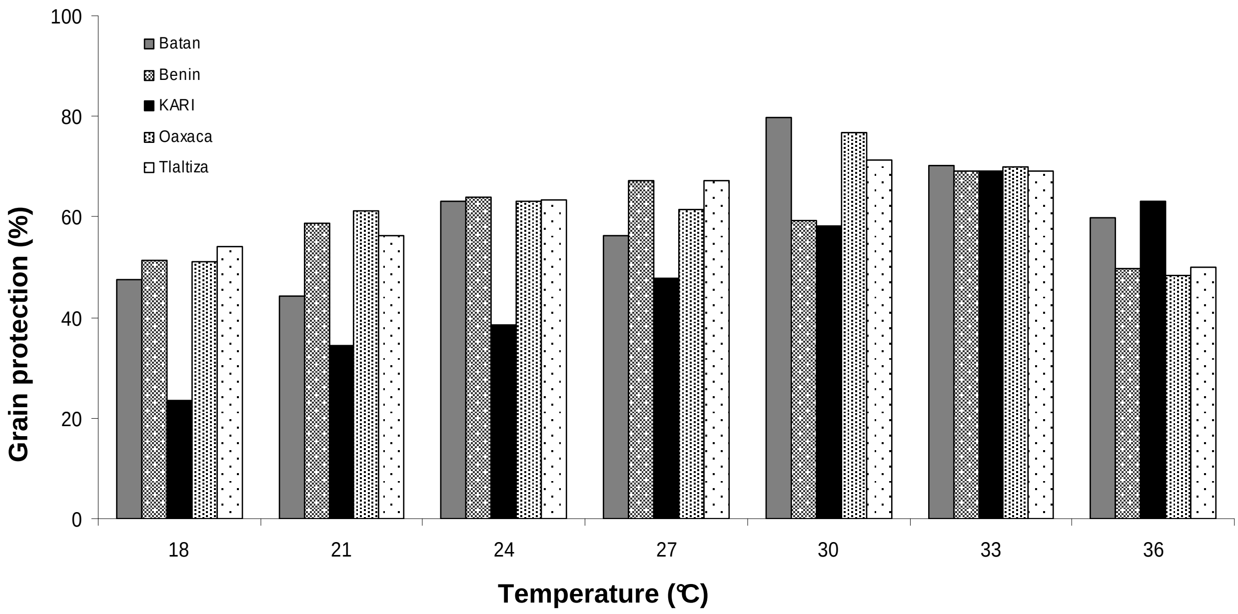

Figure 4.5: Effect of temperature on grain weight loss prevention by five strains of *Teretrius nigrescens* at 40 – 55 % relative humidity (control = 0 % protection).

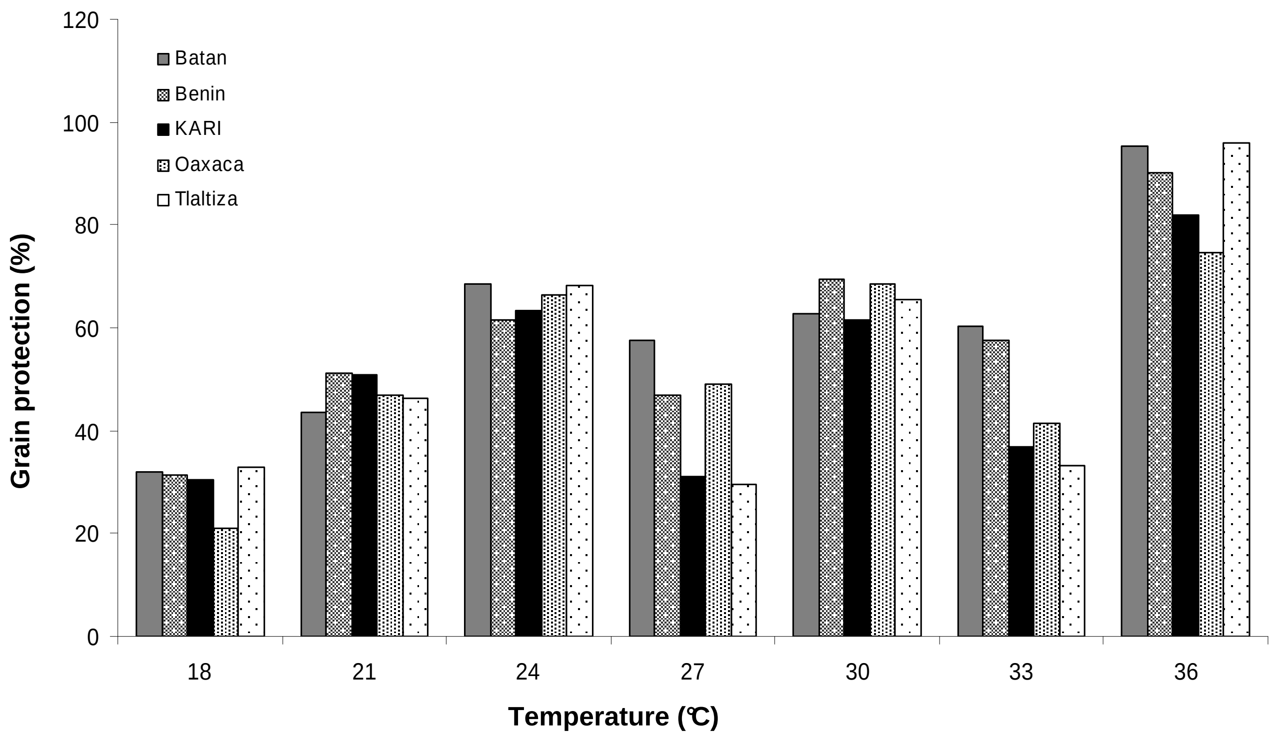

Figure 4.6: Relative effect of temperature on grain weight loss prevention by five strains of *Teretrius nigrescens* at 70 – 85 % relative humidity conditions (control = 0 % protection).

Table 4.1: Effect of temperature, humidity and *Teretrius nigrescens* strain on indicators of grain loss (mean ± sem) caused by LGB on maize grains after 70 weeks.

		Grain Weight Loss (%)		**Grain Damage (%)**		**LGB Frass produced (g)**	
		RH=70 - 85	**RH=40 - 55**	**RH=70 - 85**	**RH=40 - 55**	**RH=70 - 85**	**RH=40 - 55**
Population	**n**	**Mean ± sem**	**Mean ± sem**	**Mean ± sem**	**Mean ± sem**	**Mean ± sem**	**Mean ± sem**
				18 ˚C			
Batan	6	9.35 ± 1.85a	7.57 ± 0.65^{a}	52.01 ± 1.00^{ab}	40.01 ± 2.13^{ab}	11.96 ± 1.05^{a}	8.08 ± 0.50^{b}
Benin	8	9.56 ± 1.09a	9.62 ± 0.47^{a}	52.86 ± 1.47^{ab}	42.28 ± 1.23^{ab}	12.10 ± 0.96^{a}	9.48 ± 0.37^{b}
KARI	6	17.85 ± 4.02^{ab}	9.41 ± 0.98^{a}	55.14 ± 1.49^{bc}	41.48 ± 2.11^{ab}	21.83 ± 5.35^{a}	9.12 ± 0.76^{b}
Oaxaca	6	11.11 ± 4.25^{ab}	9.99 ± 0.62^{a}	53.67 ± 2.05^{a}	44.13 ± 1.21^{ab}	11.67 ± 1.09^{a}	9.73 ± 0.40^{b}
Tlaltizaspan	6	9.06 ± 0.89^{a}	8.23 ± 0.58^{a}	53.01 ± 0.85^{ab}	38.25 ± 1.56^{a}	13.21 ± 1.73^{a}	8.64 ± 0.51^{b}
Control	6	23.68 ± 2.59^{b}	13.44 ± 0.34^{b}	55.39 ± 1.44^{c}	46.55 ± 0.63^{b}	20.86 ± 1.51^{a}	12.17 ± 0.26^{a}
				21 ˚C			
Batan	6	15.41 ± 1.25^{a}	32.22 ± 1.98^{a}	72.74 ± 3.48^{ab}	68.13 ± 1.88^{a}	16.07 ± 0.95^{a}	22.43 ± 0.78^{b}
Benin	8	16.73 ± 0.82^{a}	29.95 ± 2.19^{a}	72.76 ± 1.44^{a}	67.34 ± 2.80^{a}	16.10 ± 0.58^{a}	22.06 ± 1.44^{b}
KARI	6	27.49 ± 5.04^{b}	29.30 ± 1.90^{a}	78.83 ± 0.63^{b}	66.64 ± 2.53^{a}	22.37 ± 4.27^{a}	21.88 ± 1.11^{b}
Oaxaca	6	13.99 ± 0.92^{a}	30.04 ± 1.25^{a}	68.09 ± 2.05^{ab}	69.15 ± 1.23^{a}	14.61 ± 0.94^{a}	22.49 ± 0.93^{b}
Tlaltizaspan	6	16.43 ± 1.07^{a}	27.33 ± 2.24^{a}	71.66 ± 2.08^{ab}	66.87 ± 1.37^{a}	15.57 ± 1.12^{a}	22.43 ± 0.78^{b}
Control	6	41.17 ± 2.14^{c}	55.28 ± 0.98^{b}	81.90 ± 1.49^{c}	82.82 ± 1.39^{b}	21.77 ± 3.13^{a}	40.22 ± 1.32^{a}
				24 ˚C			
Batan	6	23.30 ± 1.20^{a}	23.33 ± 0.83^{a}	79.50 ± 1.99^{a}	64.13 ± 1.60^{a}	20.43 ± 0.71^{ab}	16.83 ± 0.55^{c}
Benin	8	20.42 ± 0.80^{a}	27.23 ± 1.61^{b}	74.94 ± 1.86^{ab}	67.14 ± 1.84^{a}	18.62 ± 0.75^{b}	19.51 ± 1.08^{bc}
KARI	5	41.09 ± 5.21^{b}	28.47 ± 2.06^{b}	85.54 ± 0.88^{b}	68.13 ± 3.65^{a}	22.90 ± 1.17^{ab}	20.60 ± 1.45^{b}
Oaxaca	6	22.92 ± 1.66^{a}	24.50 ± 0.67^{ab}	81.00 ± 2.59^{a}	64.14 ± 0.74^{a}	20.57 ± 1.49^{b}	17.68 ± 0.57^{bc}
Tlaltizaspan	6	20.98 ± 1.61^{a}	25.19 ± 1.27^{b}	78.43 ± 1.35^{a}	66.13 ± 1.51^{a}	18.90 ± 1.11^{b}	17.87 ± 0.82b^{c}
Control	6	63.79 ± 3.65^{c}	71.31 ± 0.61^{c}	94.70 ± 0.48^{c}	87.58 ± 0.58^{b}	28.90 ± 4.83^{a}	44.12 ± 0.31^{a}
				27 ˚C			
Batan	6	25.58 ± 2.21^{a}	18.99 ± 0.59^{a}	77.45 ± 1.82^{a}	54.42 ± 1.39^{a}	26.09 ± 2.90^{b}	13.50 ± 0.46^{c}
Benin	8	22.67 ± 2.10^{a}	23.40 ± 1.50^{b}	79.73 ± 1.61^{ab}	59.96 ± 2.11^{a}	20.06 ± 1.59^{b}	16.08 ± 0.94^{bc}
KARI	6	34.57 ± 2.96^{a}	25.69 ± 2.11^{b}	84.03 ± 1.14^{b}	64.46 ± 2.19^{a}	25.31 ± 2.12^{b}	18.07 ± 1.24^{b}
Oaxaca	6	20.68 ± 2.21^{a}	21.09 ± 0.90^{ab}	77.73 ± 1.05^{a}	58.02 ± 1.04^{ab}	20.34 ± 1.15^{b}	14.92 ± 0.61^{c}
Tlaltizaspan	6	22.40 ± 3.40^{a}	24.50 ± 0.66^{ab}	77.43 ± 1.90^{ab}	61.29 ± 0.90^{a}	21.79 ± 1.52^{b}	16.13 ± 0.49^{bc}
Control	6	69.60 ± 4.88^{b}	54.63 ± 1.16^{c}	95.86 ± 0.85^{c}	80.37 ± 1.26^{c}	37.56 ± 6.47^{a}	33.88 ± 0.60^{a}
				30 ˚C			
Batan	6	16.97 ± 2.59^{a}	29.25 ± 1.24^{b}	76.94 ± 4.44^{a}	65.16 ± 1.51^{ab}	13.12 ± 1.70^{b}	19.88 ± 0.82^{b}
Benin	14	31.76 ± 3.69^{b}	24.84 ± 0.47^{b}	82.13 ± 0.99^{ab}	61.77 ± 1.05^{a}	21.40 ± 1.70^{a}	17.10 ± 0.33^{c}
KARI	11	30.05 ± 1.99^{b}	31.03 ± 1.75^{b}	86.68 ± 1.28^{b}	68.31 ± 1.41^{b}	21.21 ± 1.19^{a}	21.06 ± 1.06^{b}
Oaxaca	6	17.73 ± 1.60^{a}	24.36 ± 0.81^{a}	78.00 ± 3.26^{a}	62.81 ± 0.88^{a}	13.53 ± 1.21^{b}	16.81 ± 0.57^{c}
Tlaltizaspan	11	20.96 ± 1.59^{a}	32.02 ± 1.89^{ab}	79.21 ± 1.67^{ab}	66.45 ± 1.87^{b}	19.00 ± 1.47^{ab}	21.10 ± 0.97^{b}
Control	6	78.39 ± 1.62^{c}	79.11 ± 1.20^{c}	97.78 ± 0.48^{c}	86.52 ± 0.44^{c}	16.79 ± 2.06^{ab}	43.31 ± 0.45^{a}
				33 ˚C			
Batan	6	20.62 ± 0.92^{a}	15.83 ± 0.27^{a}	66.83 ± 2.54^{a}	34.93 ± 0.62^{a}	16.25 ± 1.28^{a}	8.06 ± 0.14^{b}
Benin	8	20.79 ± 0.93^{a}	15.39 ± 0.46^{a}	70.34 ± 2.26^{a}	33.90 ± 1.93^{a}	16.44 ± 0.75^{a}	7.74 ± 0.27^{b}
KARI	6	36.57 ± 7.12^{b}	15.92 ± 1.47^{a}	74.81 ± 3.34^{a}	38.58 ± 1.26^{b}	16.40 ± 6.61^{a}	9.36 ± 0.76^{b}
Oaxaca	6	20.49 ± 1.33^{a}	14.84 ± 0.56^{a}	69.64 ± 3.88^{a}	32.71 ± 1.24^{a}	16.28 ± 1.10^{a}	7.59 ± 0.32^{b}
Tlaltizaspan	6	19.78 ± 1.07^{a}	15.69 ± 0.21^{b}	67.72 ± 2.30^{a}	35.60 ± 0.52^{a}	15.89 ± 0.98^{a}	8.12 ± 0.11^{b}
Control	6	71.74 ± 2.95^{c}	29.94 ± 1.47^{c}	90.85 ± 0.76^{b}	48.18 ± 1.44^{b}	25.43 ± 5.50^{a}	14.36 ± 0.76^{a}
				36 ˚C			
Batan	5	10.99 ± 0.20^{a}	17.60 ± 0.37^{bc}	44.50 ± 2.82^{ab}	37.18 ± 0.82^{b}	9.00 ± 0.26^{a}	7.94 ± 0.18^{b}
Benin	5	11.30 ± 0.45^{a}	14.41 ± 0.44^{a}	47.26 ± 2.83^{ab}	29.63 ± 0.82^{a}	9.84 ± 0.40^{a}	6.69 ± 0.23^{b}
KARI	5	12.68 ± 2.26^{a}	13.87 ± 0.15^{a}	42.55 ± 1.58^{b}	29.47 ± 1.40^{a}	8.77 ± 0.46^{a}	6.47 ± 0.12^{b}
Oaxaca	6	12.54 ± 2.02^{a}	16.76 ± 0.25^{b}	50.97 ± 6.13^{ab}	35.64 ± 0.84^{b}	10.79 ± 1.76^{a}	7.49 ± 0.19^{b}
Tlaltizaspan	5	12.09 ± 0.32^{a}	17.61 ± 0.18^{bc}	47.12 ± 1.17^{ab}	36.53 ± 0.76^{b}	10.00 ± 0.30^{a}	7.63 ± 0.15^{b}
Control	6	26.47 ± 9.39^{b}	21.76 ± 1.96^{c}	59.11 ± 5.41^{a}	36.60 ± 1.35^{b}	17.56 ± 4.64^{a}	9.37 ± 0.63^{a}

Means within sub columns followed by the same letter are not significantly different (SNK test, after ANOVA; $p < 0.05$).

4.5 Discussion

Temperature influences population trends of poikilotherms through its effect on physiological activities of the organism which can be detected in its development time, fecundity and survival (Hagstrum and Milliken, 1988). The importance of relative humidity in the development of storage pests has been demonstrated by Markham (1981), Bell and Watters (1982) and Throne *et al*. (1994) and of *T. nigrescens* by Oussou *et al*. (1998). Predicting the rate of growth from ambient temperature and humidity levels is important in biological control both in their effect on the natural enemy and on the pest species. Other than rainfall, these two parameters also influence the global distribution, dispersal and establishment of new populations of pests and their natural enemies and the level of damage or control they exert (Thomson *et al*., 2009). Substrate-boring storage insects are generally adapted to minimal fluctuations in humidity and temperature conditions. Thus, estimates based on constant temperature and humidity conditions are likely to be more applicable to this category of insects. In this experiment response variables were likely to be influenced by the effect of these conditions on both species and their interaction with each other. Therefore most responses (e.g. grain damage, grain protection, frass and LGB population growth) are only compared between strains within the other two fixed variable levels or within the same temperature and different humidity levels.

Teretrius nigrescens survived all temperature levels for ten weeks, with limited population growth at extreme temperature regimes. Predation on LGB and resultant control of damage ranged between 20 % and 80 %. The apparent high grain protection index at 36 °C was most likely due to the direct negative effects of temperature on LGB survival and population growth and thus positive effect on grain damage. Under such conditions, minimal predation by the surviving *T. nigrescens* adults is likely to result in a disproportionate decline in comparative damage levels relative to the control.

Earlier workers found 26 – 30 °C to be optimum for *T. nigrescens* (Rees *et al*., 1985), corroborating the findings of the present experiments. Also, dry conditions seem to limit *T. nigrescens* more than its predator resulting in a lower index of damage control supporting the findings of Oussou *et al*. (1998). In fact, under dry conditions, there was a significant decrease in *P. truncatus* damage control exerted by *T. nigrescens* at the optimal temperature of 27 °C for almost all populations. Due to vigorous multiplication in the predator population, the shortage of prey resulting in reduced survival of the predator larvae, or a delay in maturity could have caused this decline. Disproportionately high predation resulting from rapidly rising *T. nigrescens* populations and their effect on *T. nigrescens* survivorship have been reported by Oussou *et al*. (1998) and Rees *et al*. (1990). Egg and larval cannibalism has been suspected to occur under high density rearing conditions in the absence of suitable prey (Atcha and Borgemeister, IITA rearing manual). Rapid early population growth, resulting into crowding could have similar effects if cannibalism occurs, although its effects would be greater in the second generation larvae.

At 24 °C, the Batan population was marginally superior to other populations under humid conditions, while the Oaxaca population exerted better LGB population reduction at lower than higher humidity levels. These reductions are not corroborated by other measures of survival. Again, two scenarios are possible. A voracious predator population possibly exerted greater control of the prey, eventually limiting its own population build-up. This results in the eventual observation of low numbers of both species. This scenario is suspected for the Benin and Oaxaca population at 30 °C. A second possibility, also associated with inconsistent trends observed under high humidity, is the sex ratio of the predator in each set up. *Teretrius nigrescens* cannot be sexed using observable secondary features (Böye, 1988). Consequently, the 20 insects starting culture could have had varying proportions of females, with a resultant variation in the contribution of each starting individual to the population counted at the end of the experiment. This random event is however more likely to increase the variation of observations within treatments across the all populations than to be more pronounced at a specific temperature level. In both cases, however, it would be inconclusive to compare the predator populations from these data without conducting single predation and development experiments, which would include post experimental sex determination.

Extreme damage levels were observed in this experiment. Between 24 °C and 30 °C, there was near total destruction of the grains with most damaged kernels barely discernible. Such levels of damage are unlikely in the storage environment in such a short time and were the result of a high initial infestation level. The extreme damage of maize grain could also have limited LGB development and hence *T. nigrescens*. Lower LGB infestation rates would aggravate the effects of sex ratio differences between treatments and replicates, while higher maize quantities would significantly increase the time required to dissect and count insects from the grains, which poses logistical limitations.

The hypothesis that biotypes of *P. truncatus* and *T. nigrescens* exist has been invoked to explain the variation in pest status and performance of the predator under different agro-ecological zones (Nansen and Meikle, 2002). Geographical populations of the pest have shown significant variation in fitness and phenotypic traits including development time, longevity, oviposition rate fertility, enzyme activity and diversity of gut flora (Guntrip *et al*., 1996; Vazquez-Arista *et al*., 1999; Nansen and Meikle, 2002). Such variation has not been clear in *T. nigrescens*. Anecdotal evidence shows that a comparison of mid-altitude Mexican *T. nigrescens* and the Costa Rican population originally released in West Africa did not reveal significant variation in their response to temperature variation (Anonymous, 1999). However, the hypothesis that there are different populations of *T. nigrescens* adapted to different climatic conditions, which would affect their efficiency, could not be conclusively tested by this study. Complete life table studies, or at least studies of the effect of temperature and humidity conditions on the developmental, fitness phenotypic characteristics would be necessary.

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CHAPTER FIVE

Phylogenetic relationship between ten populations of *Teretrius nigrescens*

5.1 Abstract

Molecular markers are a powerful tool for studying population genetics and phylogenetics. They may reveal details about population sources, migration history and evolutionary events. Data from *Teretrius nigrescens* monitoring studies and results and physiological tests, suggest that ecological or geographical strains of do exist. This study sought to identify phylogentic relationships between geographical populations of *T. nigrescens* and to design molecular markers for the identification any possible trains. Ten populations of *T. nigrescens* were studied using RAPD-PCR, sequence analysis of ribosomal ITS1, 5.8S and ITS2 and mtCOI from 10 populations. The mtCOI variation revealed two clades associated with geographical regions in Central America. It also indicated considerable reduction in the genetic diversity in laboratory cultures. RAPD-PCR did not reveal any potential SCAR markers. The ITS variation mainly involved insertion and deletion (indels) of simple sequence repeats even within individuals. This study concludes that there exist two parapatric mitochondrial lineages of the predator associated with the geographical origin of populations. However, the study suggests the inclusion of more variable markers to clarify the observations in demographic time scale.

5.2 Introduction

The success of biological control of the larger grain borer (LGB) by *T. nigrescens* Lewis in Africa has been largely dependent on the strain used and the ecological conditions in the area of release (Meikle *et al*., 2002; Schneider *et al*., 2004). This led to the hypothesis that the strains released would only succeed where environmental conditions were suitable. The low pest status of the LGB and presence of the predator in a wide range of environmental conditions in Africa led to the hypothesis that ecotypes exist. However, a review of the literature does not reveal distinct discontinuity, among geographical populations making it difficult to estimate the level of differentiation that would be expected among and between the populations. Knowledge of the genetic structure of *T. nigrescens* populations has potential use in guiding the exploration for new populations, monitoring released populations and their interactions with the environment, other populations and the pest.

Molecular markers have become invaluable in ecological and evolutionary studies. In entomology particularly, DNA markers provide a means of identification of organisms where morphological differences are not obvious or where taxonomically important stages or characters are not clear (Loxdale and Lushai, 1998). Despite these attributes, the choice of the marker to apply in a specific situation is sometimes subjective, as a result of the variation in evolution rates of specific loci (Caterino *et al.*, 2000). The challenge is to find a gene that is variable enough to reveal significant differences, yet conserved enough to be meaningfully analysable.

Mitochondrial DNA (mtDNA) is now commonly used in phylogenetic and population genetic studies. It is matrilineally inherited, expressed, nearly conserved in function and arrangement across many taxa and evolve much faster than the nuclear genes (Hwang and Kim, 1999). Therefore, gene arrangement, sequence variation and protein sequences can all be used at different levels of taxonomic studies. The existence of universal primers for mitochondrial genes enables the amplification of loci without detailed prior sequence information (Simon *et al.*, 1994; Zhang and Hewitt, 1997). The subunit 1 of the mitochondrial cytochrome oxidase gene (mtCOI) is widely used due to variation in the rate of evolution in different regions (Lunt *et al.*, 1996). Coding genes, however, have a greater restriction to applicability in differentiating between populations.

The internal transcribed spacer (ITS) regions provide a powerful tool in population and phylogenetic studies since both hypervariable nucleotide sequence and less variable folding pattern of the initial RNA transcript of these sequences can be studied (Müller, 2007; Coleman, 2009). The nuclear ribosomal DNA (rDNA) consists of bundles of tandem arrays of genes of high copy number, containing both coding and non-coding regions (Zhang and Hewitt, 2003). Nuclear rDNA spacer regions evolve much faster than coding regions since mutations are not limited by fitness constraints. Nuclear DNA undergoes genetic recombination resulting in possible nucleotide and length variation within individuals and populations. The process of concerted evolution somewhat corrects these difference making certain sequences more or less fixed within breeding populations (Hwang and Kim 1999; Zhang and Hewitt, 2003). This makes rDNA useful in studying the mating interactions between populations (Fritz *et al*., 1994; Zhang and Hewitt, 2003).

The separate introduction of two populations of *T. nigrescens* into Africa, isolated over 800 kilometres apart, raises the possibility that distinct strains were involved. Both strains however were not effective in controlling the larger grain borer in warm dry climates (Giles *et al*., 1996; Hill *et al*., 2003; Hodges *et al*., 2003; Schneider *et al*., 2004). After extensive surveys using pheromone traps, Tigar *et al.* (1994) modelled the conditions responsible for the survival of both species in Mexico and inferred that they could exist in a wide range of regions. Rees *et al*. (1990) recovered both LGB and *T. nigrescens* under very warm, dry conditions in the Yucatan Peninsula. It is thus possible that the limitations of the *T. nigrescens* populations introduced into Africa are partially due to their inherent genetic limitations. To address this problem, four populations of the predator from Mexico and Honduras have been imported into the quarantine facilities of ICIPE, Nairobi, for eventual release. Pertinent questions still to be answered are: What is the extent of genetic variation in *T. nigrescens*? Do geographical populations of *T. nigrescens* represent distinct ecotypes? Can molecular markers be used to distinguish between these ecotypes? How can this genetic diversity be incorporated into a biological control program? To investigate this questions, the sequence variation of mtCOI gene and both ITS1 and ITS2 spacer regions among the populations of the predator was studied (Table 2.1).

5.3 Materials and Methods

5.3.1 DNA extraction

Total genomic DNA was isolated from the heads and thoraxes of individual adult *T. nigrescens* using the Phenol-Chloroform protocol (Sambrook *et al*., 1989). DNA was stored in TE buffer (10 mM TrisHCl pH 8, 1 mM EDTA) at -20 °C until ready for use (Chapter 2). For out-group species, single legs were used and the rest of the insect submitted for identification. Insect identification to species level was done by Prof. Pierpaolo Vienna, Venezia Lido, Italy.

5.3.2 Polymerase Chain Reaction

RAPD-PCR trials

Random amplified polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) (Welsh *et al*., 1990; Williams *et al*., 1990) was used to screen the wide diversity within and among geographic populations. The KARI, Oaxaca, Tlaltizaspan, El Batan and Benin geographic populations were used in this study representing the widest geographical range of the populations available. Twenty five decamer random primers previously used in insect genetic diversity studies (Moya *et al.*, 2001; Omondi *et al.*, 2004) were screened for polymorphism, and reproducibility. Thirteen of these were selected and used to amplify DNA from samples across populations.

The PCR reaction was done in a 25 µl mix containing 1x Genscript *Taq* polymerase buffer with 3.0 mM $MgCl_2$, 400 µM dNTPs, 300 nM single RAPD Primer, 1U *Taq* polymerase and ~ 20 ng template DNA. The amplification was done in an ABI 9800 thermal cycler programmed as follows: one cycle of 94 °C for 3 min, followed by 35 cycles of a minute each of 94 °C denaturation, annealing at 35 °C and extension at 72 °C, and a final extension step of 72 °C for 10 min. Products were held at 4 °C till ready for use. The amplicons were separated on 2 % agarose (in TAE) gels stained with ethidium bromide under 2.5 volts/cm potential gradient for 2 hours. Gels were visualised under UV light. Since there were no bands for potential use as to develop sequence characterised amplified region (SCAR) markers, RADP PCR was not pursued further.

Cytochrome Oxidase 1

A 1200 bp region of the mtCOI gene, was amplified by PCR using the universal insect mtCOI: C1J-17363 (5´ TATAGCATTCCCACTAATAAATAA 3´) forward and TL2-N-3014 (5´ TCCAATGACTAATCTGCCATATTA 3´) reverse primers previously described by Zhang and Hewitt (1997). The PCR amplifications were carried out in 30 µl reaction volumes containing 1x Genscript *Taq* polymerase buffer with 1.5 mM $MgCl_2$, 400 µM dNTPs, 150 nM each of the forward and reverse primers, 1U *Taq* polymerase and ~ 20 ng genomic DNA. The thermal cycling programme was 94 °C 3 min, followed by 35 cycles of denaturation at 94 °C 1 min, annealing at 52 °C for 40 s and extension at 72 °C for 90 s, and then a final extension step of 72 °C for 10 min. The PCR product was separated on a 1.2 % agarose gel. The amplification product of 1200 bp size was excised from the gel and purified using Quiagen Min Elute Gel purification kit (Quiagen) according to manufacturer’s instructions (Chapter 2). The eluted product was sequenced in both directions using the PCR primers on an ABI chain termination sequencing technology at a commercial facility (Macrogen Inc, Korea). (Chapter 2)

Internal transcribed spacer regions (ITS)

Amplification of the two ITS regions and 5.8S rRNA coding site was done as above using standard forward primer ITS5 (5´ GGAAGTAAAAGTCGTAACAAGG 3´) and reverse primer ITS4 (5´ TCCTCCGCTTATTGATATGC 3´) (White *et al*., 1990). The thermocycling conditions were similar to those of mtCOI (above) but with an annealing temperature of 54 °C and running length of 25 cycles. Fewer cycles were used to increase the integrity of the product for cloning. Preliminary direct sequencing of ITS genes produced equivocal results; hence cloning was done to reveal possible intra-genomic variation.

The gel purified fragment was cloned into pGEMT-E vector (Promega) and used to transform DH5α chemically competent cells as already described (Chapter 2). Recombinant colonies were screened by PCR to confirm the presence of the insert. Five positive recombinant colonies per insect sample were selected for bacterial propagation and plasmid purification. Plasmid purification was done using Quiagen Miniprep Kit according to manufacturer's protocol. Plasmid and DNA samples were sequenced by the ABI terminator system at Macrogen Inc. Korea using M13FpUC and M13RpUC universal primers (Promega) at Macrogen Inc.

5.3.3 PCR-RFLP analysis

Sequence alignment revealed parsimony informative segregating sites, associated with populations, so, NEB CUTTER version 2 (Vincze *et al*., 2003) software was used to predict the diagnostic utility of the restriction endonucleases cleaving parsimony informative sites. The selected restriction enzymes were then used to digest PCR products of the mtCOI from the five populations of *T. nigrescens* (Ghana, Oaxaca, Benin, KARI and Tlaltizaspan) representing the main sub clusters in sequence analysis.

5.3.4 Data Analysis

DNA sequences were edited manually to confirm the positions of ambiguous nucleotide calls using BIOEDIT (Hall, 1999). The termini of the sequences were trimmed to remove regions of unreliable sequencing quality. The reverse complement of the reverse sequence was contiged with the forward sequence after pair wise alignment of the termini produced at least 100 bp complete consensus overlap region. All complete sequences were aligned together and segregating sites reconfirmed by comparison with the trace diagrams and translation using the Invertebrate Mitochondrial codon table. Since mtCOI is an expressed gene, only fragments translating into a complete reading frame protein after sequence confirmation were considered. This would avoid error from possible nuclear mitochondrial pseudogenes (Bensasson *et al*., 2000). Haplotype identification and frequencies were done manually by sequences alignment and grouping of sequences according to similarity in BIOEDIT. Haplotype network was determined in TCS version 2.1 (Clement *et al*., 2000). Haplotypes were grouped into series of clades from the smallest group of individual haplotypes. Due to the distinct separation of three clades (at least 22 steps), and rearing history of laboratory colonies, population inferences were based on the haplotype network of trees rather than a statistical nested clade analysis.

Multiple sequence alignment was performed using Clustal W version 1.8 as implemented in BIOEDIT. The numbers and types of segregating sites, Kimura 2-parameter distances (K2P) between individuals, populations and groups of populations were determined using MEGA 4.0 (Kumar *et al*., 2001). In this approach, the mean number of base substitutions per site from all 59 sequences and 1084 bases per sequence was conducted using the Maximum Composite Likelihood method in MEGA4 (Tamura *et al*., 2004; 2007). Average within and between geographic population K2P distances and their standard deviation were calculated using MEGA 4.0. Due sequence similarity among samples from Honduras were combined into one population. In determining the genetic diversity between groups of populations, the 'Store' Population was analysed separately since its antiquity could not be ascertained.

The phylogenetic relationships between the samples were inferred using a neighbour-joining tree on MEGA 4 (Saitou and Nei, 1987). Sequence alignment was done in Clustal X to create an alignment file for MEGA. The evolutionary relationships were inferred with Neighbour-Joining tree method, with 1000 replicates. The evolutionary distances used to compute tree-branch length were determined. Similar length mtCOI sequence from *Teretrius americanus* LeConte (= *T. latebricola* Lewis) was used as an out group species.

The ITS1 and ITS2 sequences were aligned manually on BIOEDIT, since, due to numerous indels, automatic alignment was not satisfactory. The repeat regions were identified manually from the BIOEDIT alignment and using SSR Finder (Benson, 1999). The boundaries of the 18S, 5.8S and 21S rRNA genes were delimited based comparisons with sequences of *Timarcha* and *Tetranynchus* sequences (Navajas *et al*., 1998; Gomez Zurita *et al*., 2000).

5.4 Results

RAPD-PCR profiles obtained using all the 13 primers were reproducible with numerous polymorphic bands (Figure 5.1). However the polymorphism of profiles was not associated with any geographical or demographic historical characteristics of the populations.

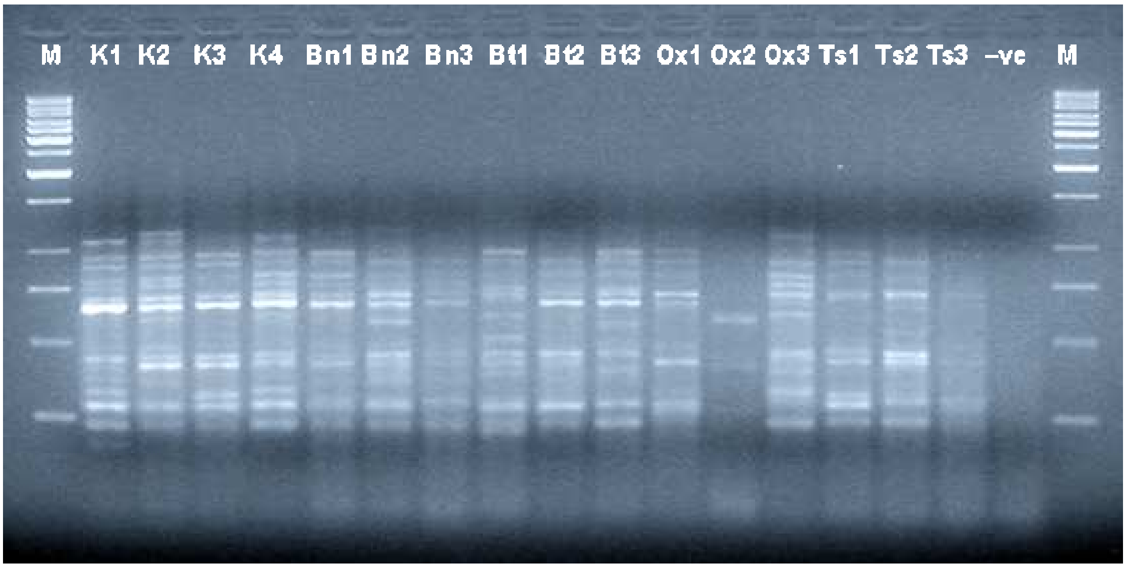


Figure 5.1: RAPD PCR profiles of four populations of *Teretrius nigrescens* from primer OPB 11 run on 2% agarose (in TAE) gel. Population used are: K - Kari, Bn - Benin; Bt - Batan; Ox - Oaxaca and Ts - Tlaltizaspan. M represents 1 kb DNA ladder.

5.4.1 MtCOI Sequence variation

A 1200 bp region was amplified with the mtCOI primers. After editing, a 1084 bp fragment was used in the analyses whose results are presented below. The mtCOI sequence was 63.4 % A/T rich. The nucleotide frequencies were 0.285 (A), 0.349 (T), 0.203 (C), and 0.163 (G), comparable to mitochondrial sequences in other Coleoptera (Cognato *et al*., 2003; Mynhardt *et al*., 2007). There were 102 polymorphic sites, of which 72 were parsimony informative (Figure 5.2). This variation occurred most at third (83.3 %), first (13.7 %) and second (9 %) codon positions. The rate and direction of nucleotide substitutions is summarised in Table 5.1. No insertions or deletions were observed among *T. nigrescens* samples or species of Histeriae used as out groups.

5.4.2 Genetic diversity

Pair-wise divergence between populations based on K2P parameter are shown in Tables 6.2 and 6.3. There was greater evolutionary divergence among samples from the Honduras/Costa Rica than there was from the samples from Mexico. However, the Mexican Tlaltizaspan population was closest to individuals from Ghana and Malawi both originating from the Costa Rica population reared and released in Benin.

The evolutionary relationships between the 57 *T. nigrescens* samples revealed two major clades supported by > 98% bootstrap values (Figure 5.3). One cluster contained most samples originally recovered from Mexico, while the other grouped samples from Honduras, Costa Rica and Tlaltizaspan (Mexico). Other minor clusters were highly supported too (e.g. all Store samples). These grouped samples from highly monomorphic populations or single haplotype. Twenty-two haplotypes were observed of which 15 were unique (observed in only one individual each) (Figure 5.2). Haplotype diversity was highest among the Honduras populations (7 haplotypes) and lowest among the KARI, Store and Malawi populations (2 haplotypes each) (Figure 5.4). Haplotype H19 was both the most diverse and the most frequent, occurring in five of the populations and 23 % of the individuals (Figure 5.5).

5.4.3 PCR-RFLP identification of populations

In silico restriction analysis showed that the restriction endonucleases *Aci*I, *Bst*YI, *Dde*I, *Fok*I and *Rsa*I, had their recognition sites flanking parsimony-informative sites and so could resolve four populations into two major clades and (Table 5.4). The results of these digestions are shown in Figure 5.6.

Variable positions marked from the beginning of the 1083 by fragment

```
                                                                                                            111111 11
           1111 1111122222 2222233333 3333344444 4444444555 5555666666 66667777776 77788888887 88899999998 9999000000 00
     2456691123 4677745566 7789901112 4468900123 4457789035 7888001112 58890124565 78912224477 79900023447 4699024556 66
     7213931235 4247865614 0424761587 2831628400 1475842112 6358392587 51463164455 43292463944 91736873259 8906507366 78
H8   GAATATTGTA AGATTCACGT ACTCGGCTCA TTCGTCGGCA CTCTCTGCCA ACTAATATAT TAGTTTTTGT AACGTCCTTT AGCCCTGGTT ATTCACTAAA GT
H10  .......... .......... .........G .......... .......... .......... .......... .......... .......... .........G ..
H9   .......... .......... .........G .......... .......... .......... ..A....... .......... .......... .......... ..
H12  ..G.....C. .AG.C.GT.. ...TT..... ...A.TAA.. ....T..T.. .....C.... C....C.C.. ...A...... ........C. .......... AC
H15  ..G....TC. .AG.C.GT.. ...TT..... ...A.TAA.. ....T..T.. .....C.... C....C.C.. ...A...... .....C..C. ....G..... AC
H13  ..G.....C. .AG...GT.. .......... ...A.TA... ....T..TT. .....C.... CG...C.C.. ...A..T... ...T...AC. ..C....... AC
H14  ..G.....C. .AG...GT.. .......... ...A.TA... ....T..TT. .....C.... CG...C.C.. .......... ...T...AC. ..C....... AC
H3   A.G..A...C GA......AC G....A.... ........T. ....TCA... ....GCT... C.ACCC.C.. .G.A.AT... TA....A... T......... AC
H4   A.G..A...C GA......AC G....A.... ........TG ....T.A... ....GCT... C.ACCC.C.. .G.A.A.... TA....A... ......A... AC
H1   A.G..A...C GA......AC G....A.... ........T. .C..T.A... ....GCT... C.ACCC.C.. .G.A.AT... TA....A.C. .......... AC
H2   A.G..A...C GA......AC G....A.... ........T. .C..T.A... ....GCT... C.ACCC.C.. .G.A.AT... TA....A... .......... AC
H11  ..G..A...C GA......AC G......... ........T. .C..T.A... ....GCT... C.ACCC.C.. .G.A.AT... TA....A... .......... AC
H7   A.G..A...T GA......AC G......... .C......T. ....T.A... ....GCT.G. C.A.CC.C.. .G.ACAT... TA....A... .....T.... A.
H5   A.G..A...C GA.......C G......C.. ........T. ...CT.A..G ..CGGCT... C.A.CC.C.C .G.ACATC.. T.....AAC. .......... AC
H6   A.G..A...C GA.......C G......... ........T. ...CT.A... ..CGGCT... C.A.CC.C.C .G.ACAT... T.....A.C. .......... AC
H16  .GGCCAC..C .A.C.T..A. GAC...T.T. ACT.....T. ....T..... ....GCT.G. C.A.CC.C.. .G.ACAT... TA....A... .....T...G A.
H18  .GGCC.C..C .A.C.T..A. ..C..AT.TG ..T.A...T. T.T.T...TG TG...C.C.C C.A.CCCCA. G.TACT..CC TAT.T.AACC .C.T.....G .C
H19  .GGCC.C..C .A.C.T..A. ..C..AT.TG ..T.A...T. T.T.T...TG TG...C.C.C C.A.CCCC.. G.TACT..CC TAT.T.AACC .C.T.....G .C
H20  .GGCC.C..C .A.C.T..A. ..C..AT.TG ..T.A...T. T.T.T...TG TG...C.C.C C.A.C.CC.. G.TACT..CC TAT.T.AACC .C.T.....G .C
H21  .GGCC.C..C .A.C.T..A. ..C...T.TG ..T.A...T. T.T.T...TG TG...C.C.C C.A.CCCC.. G.TACT..CC TAT.T.AACC .C.T....TG .C
H22  .GGCC.C..C .A.C.T..A. ..C...T.TG ..T.A...T. T.T.T...TG TG...C.C.C ..A.CCCC.. G.TACT..CC TAT.T.AACC .C.T...G.G .C
H17  .GGCC.C..C .A.C....A. ..C..AT.TG ..T.A...T. T.T.T...TG TG...C.C.C C.A.CCCC.. G.TACT..CC TAT.T.AACC .C.T.....G AC
```

Figure 5.2: Nucleotide variation among 22 mtCOI haplotypes (H1 to H22) on 1084 bases of the mtCOI *T. nigrescens* used in this study. Numbers at the top of each nucleotide position represent the segregating site position relative to the first nucleotide of this fragment. Haplotypes are arranged in order of relative similarity. Dots represent similarity with haplotype H8 while the nucleotide in each segregating site is labeled vertically (such that the first is 27 bp and the last is 1068 bp from the origin).

Table 5.1: Maximum composite likelihood estimate of the pattern of nucleotide substitution.

	Nucleotide			
	A	**T**	**C**	**G**
A	-	2.33	1.36	12.89
T	1.9	-	18.87	1.09
C	1.9	32.39	-	1.09
G	22.5	2.33	1.36	-

Each entry shows the probability of substitution from one base (row) to another base (column).

Table 5.2 Estimates of evolutionary divergence over sequence pairs between populations

Population	KARI	Benin	Kiboko	Oaxaca	Honduras	Batan	Store	Malawi	Ghana
Benin	0.0254								
Kiboko	0.0216	0.0287							
Oaxaca	0.0087	0.0281	0.0248						
Honduras	0.0417	0.0366	0.0361	0.0379					
Batan	0.0022	0.0277	0.0239	0.0109	0.0441				
Store	0.0432	0.0386	0.0344	0.0387	0.0226	0.0456			
Malawi	0.0473	0.0317	0.0354	0.0425	0.0216	0.0497	0.0232		
Ghana	0.0527	0.0405	0.0428	0.0483	0.0264	0.0552	0.0299	0.0261	
Tlaltizaspan	0.0523	0.0321	0.0399	0.0490	0.0302	0.0548	0.0356	0.0219	0.0213

Table 5.3: K2P distances between pairs of major population clades of *Teretrius nigrescens*. “Store” is treated separately as the exact source is unresolved.

	Mexico	Benin
Mexico	0.0000	
Benin	0.0418 ± 0.0049	0.0000
Store	0.0398 ±0.0052	0.0303 ± 0.0036

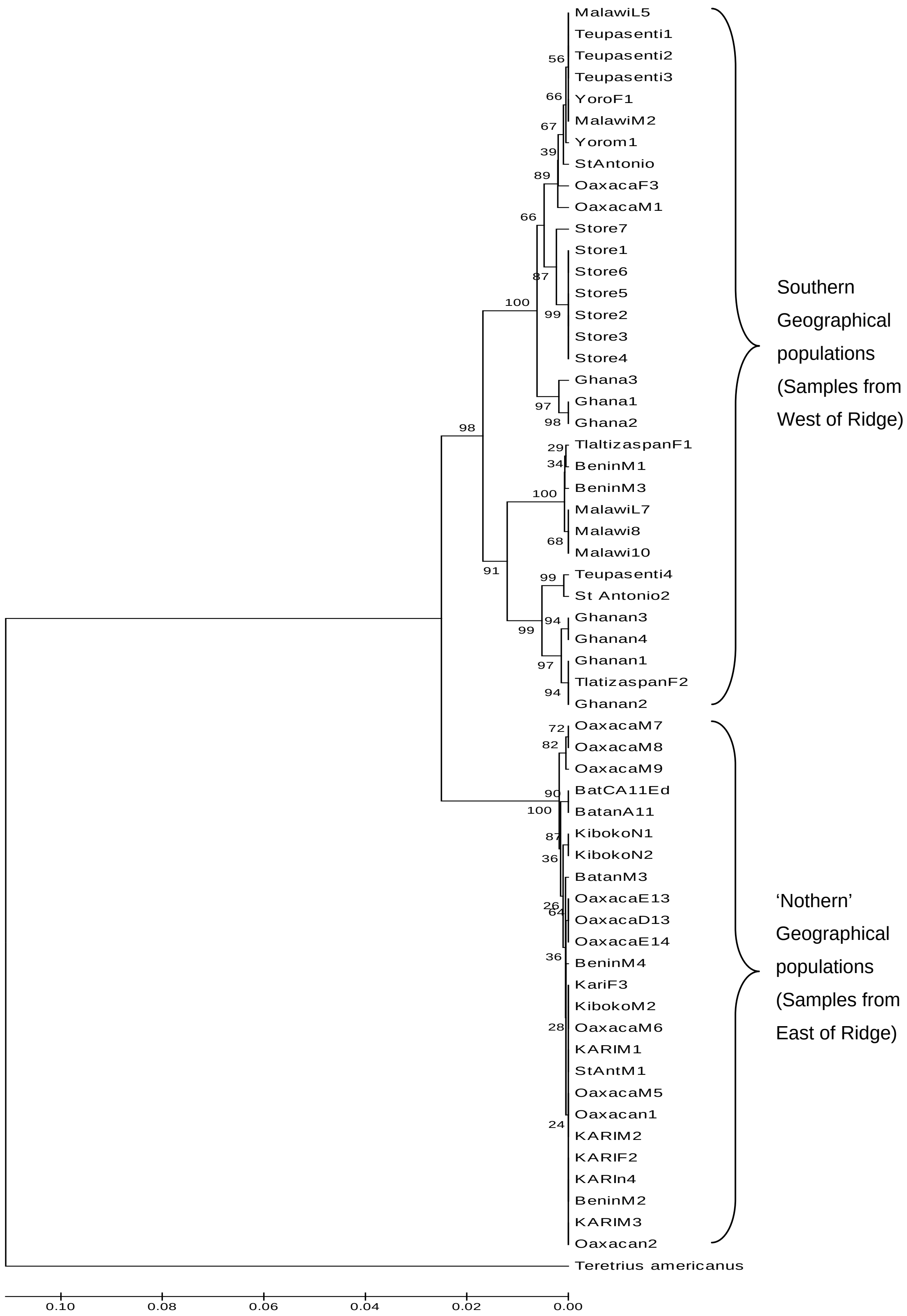


Figure 5.3: Evolutionary relationships of 57 samples of *T. nigrescens* from seven populations analysed using UPGMA method. The tree is rooted using a homologous sequence from *T. americanus.* This bootstrap consensus tree inferred from 5000 replicates is shown, with the bootstrap values next to the branches

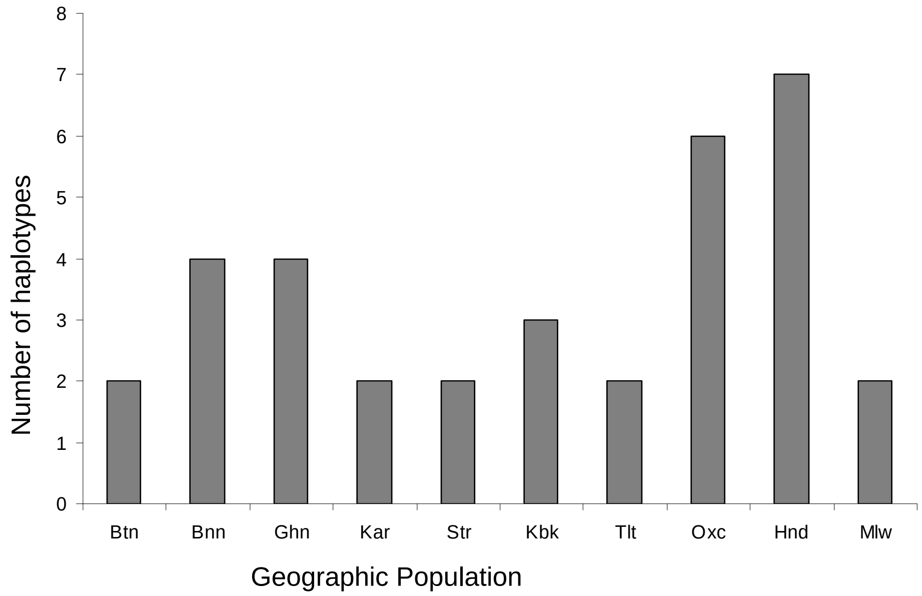


Figure 5.4: Haplotype frequency of different populations of *T. nigrescens*. Btn- Batan; Bnn- Benin; Ghn - Ghana, Kar – KARI, Tlt -Tlatizaspan; Oxc – Oaxaca; Hnd- Honduras; Mlw – Malawi

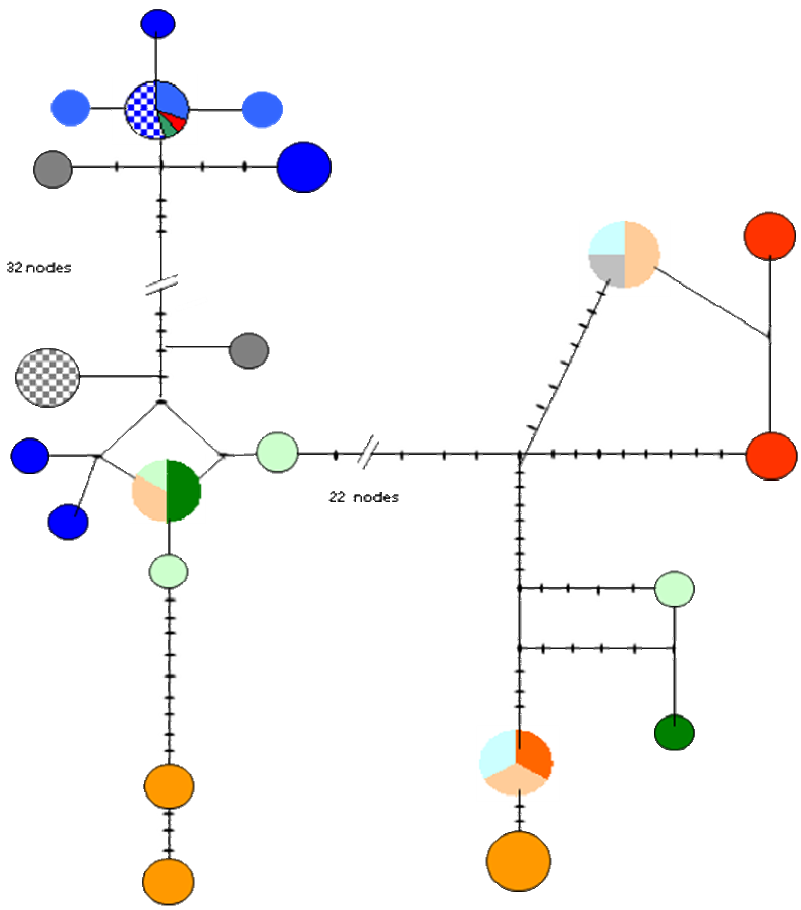
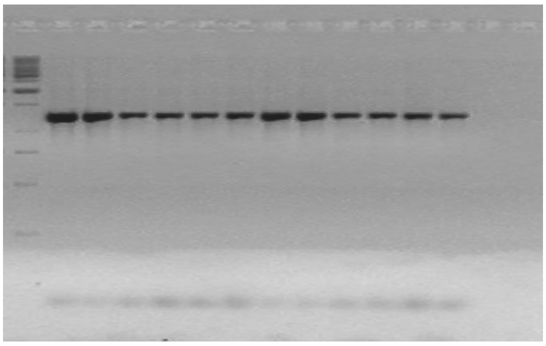


Figure 5.5: Network showing the most parsimonious relationship between 22 mtCOI haplotypes of *T. nigreescens* from 10 geographical populations. Circles represent haplotypes, black dots signify intermediate (unsampled) haplotypes and lines between haplotypes and denote single nucleotide change. Major colour shades viz: Blue – Mexican populations; Red/Orange (Costa Rican Populations); Green; Honduras populations and grey (“Field” or “Store” populations with unidentified source).

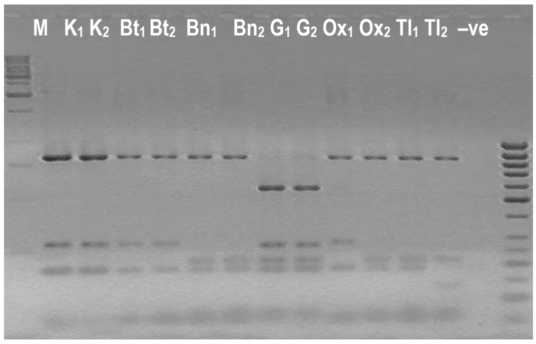
Table 5.4: Predicted products of the cleavage of 1200 bp mtCOI PCR product using four potentially diagnostic endonucleases[1].

Enzyme	Fragments Produced		Remarks
	Mexico	**Costa Rica**	
***Rsa*I**	39, 432, 701,	432, 740	Good
***Fok*I**	123, 510, 598	124, 213, **443**, **451**	Fair
***Bst*YI**	59, 475, 697	475,755	Good
***Aci*I**	59, 469, 703	**199, 200**, 832	Good
***Dde*I**	123, 212, 869	124, **267**, **270**, 570	Fair

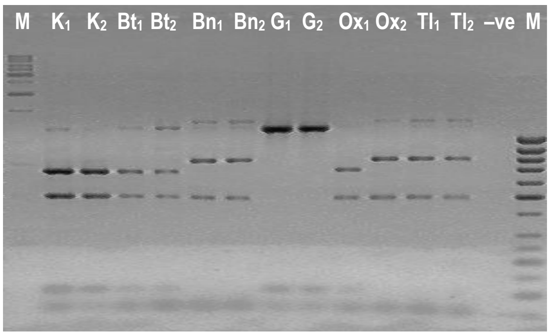
[1]Fragments in bold, for each enzyme, are not distinguishable on the agarose gel



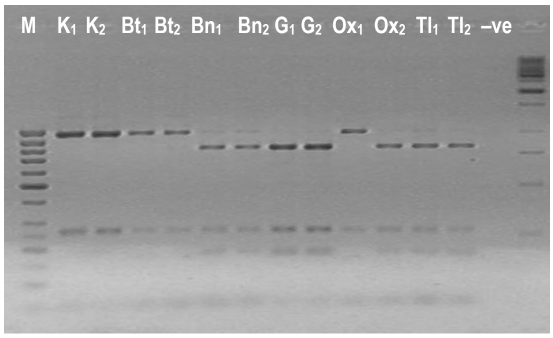
A: 1200 bp PCR product



B: Digestion with *Acc*I



C: Digestion with *Rsa*I



D: Digestion with *Dde*I

Figure 5.6: The ≈1200 bp amplicon and products of its single digestion with three restriction enzymes. Sample abbreviations are: K - KARI; Bt: Batan; Ox: Oaxaca; G – Ghana, Tl - Tlatizapan; '-ve': negative control set up without DNA.

5.4.4 Internal transcribed spacer regions

Cloning enabled the sequencing of the whole of the ITS amplicons with flanking coding sequences. This enabled comparison between samples and outgroup species. The length of the ITS sequences ranged between 1250 and 1350 bp. Intragenomic variation of up to 10 bases were observed. Most of this variation involved insertion and deletion of tandem sequence repeat units (Appendix A). Differences were therefore in multiples of repeat lengths. Because of little variation in the non repeat sequences and high polymorphism in the repeat variable regions, the ITS was not found useful for the phylogenetic analysis in this study, without major editing to remove variable parts. This gene was therefore not considered further in the phylogenetics and identification of the populations of *T. nigrescens.*

5.5 Discussion

Of the three methods used in this study, the mtCOI sequences were the most useful in the detection of population genetic variations in *T. nigrescens*. The RAPD-PCR technique used in preliminary analysis could not detect variations between populations. The RAPD-PCR technique amplifies fragments whose location in the genome is unknown (Loxdale and Lushai *et al*., 1998). Although the technique suffers technical reproducibility limitations (Lynch and Milligan, 1994) these can be minimized by optimizing reaction conditions and careful selection of primers to use. The RAPD-PCR technique has been used to differentiate between biotypes and detect interbreeding between populations of insects (Moya *et al*., 2001; De Barro and Hart, 2000; Omondi *et al*., 2005). This technique has also been used for the development of diagnostic sequence characterized amplified region sequence (SCAR) markers from by sequencing unique fragments (Rugienius *et al*., 2006). Such studies depend on prior identification of fixed differences in RAPD profiles in these populations (Omondi *et al*., 2004). Although amplifications were repeatable, there were no population-specific markers. This was attributed to three possible causes: the insects might have been too closely related genetically to have significant differences in their DNA detectable by this procedure; the primers used were simply not amplifying variable loci genetic populations were possibly sympatric at sampling locations and could not be defined by geographical sampling approach of this study. In the first two cases, greater primer screening would have revealed useful markers amplifying sites variable between populations.

The ITS 1 and ITS 2 sequences showed comparable sizes and coding region similarity with the previously studied beetle species (Gomez-Zurita *et al.*, 2000). Since the variation was predominantly due to 2 or 3 base pair tandem repeats it might be interesting to investigate how the repeat-length fit with microsatellite models in population genetic analysis. However, one must first resolve the apparent polyploid genotype pattern created by the intragenomic variation of these markers.

The mtCOI gene sequence analysis revealed two major clades of *T. nigrescens* associated with defined geographical ranges. Apart from the "Store" samples whose origin was not clearly known, all insect samples from Africa clustered with their original source populations confirming suitability of this marker for phylogenetic analysis (Otranto *et al*., 2003). The 1200 bp region sequenced in this study covered two highly variable regions and a much more conserved portion of this gene (Lunt *et al*., 1996; Zhang and Hewitt, 1997). This strategy promised greater accuracy since the phylogenetic differentiation of this species was initially unknown. Several regions of the sequence with a greater density of parsimony informative variable sites have been identified in this study. Shorter fragments spanning these desirable regions would be more practical, to reduce the potential analysis challenges associated with a large number of single sequence haplotypes and potential error in sequencing large fragments. Although very few parsimony-informative segregating sites are within 5 bases of each other, careful primer design might reveal a single step multiplex PCR protocol for identifying these populations (cf. Lindell and Murphy, 2008).

The 22 haplotypes separated into two clusters, suggesting the existence of two distinct maternal lineages, suggesting historical geographical and hence genetic fragmentation. This could have impeded interaction between populations causing independent evolution of this gene in the two populations. This study identified the region between Oaxaca and Tlaltizaspan as the possible contact zone where both subtypes exist. Lindell and Murphy (2008) have identified the Baja peninsula in north-western Mexico as a possible hybrid zone mitochondrial forms of Lizard *Uta stansburiana,* Baird & Girard. This hybrid zone was explained to reflect a complex of geological history events since the Miocene era (Lindell *et al*., 2006). Although all samples of *T. nigrescens* were obtained south of the Baja peninsula, the extent of the effects historical geological disturbances would vary for individual species depending on their original distribution and the effect of geological events on their eventual dispersal. Thus, a study of the geological and ecological history of the natural range of *T. nigrescens* would clarify this possibility. This was beyond the scope of the study, however. Alternatively, the *T. nigrescens* could have spread north and southwards from the zone around Oaxaca, founding the two different clades. This hypothesis is supported by the haplotype differentiation.

There is a possibility of population fragmentation as suggested by a high frequency of unique haplotypes only shared among a few individuals within specific populations. The effect of local geographical features may also be responsible for the variation and lineage divisions observed in this study. It is recognized that the adaptability of an invader, rather than sheer genetic diversity or numbers, are responsible for the establishment of a species in the new range (Lloyd *et al*., 2005; Zayed *et al*., 2007; Bacigalupe, 2008). However, adaptability is also a factor of the diversity of the gene pool introduced upon which selection occurs to produce resulting in an adaptable population (Roderick and Navajas, 2004). The chances of establishment and range expansion may therefore be higher with an increased genetic base (Kolar and Lodge, 2001; Allendorf and Lunquist, 2001; Phillips *et al*., 2008). Adequate sampling for a greater genetic pool is important in recovery of natural enemies (Omwega and Overholt, 1996; Phillips *et al*., 2008) and should guide future sampling plans for a more genetically inclusive approach to the biological control of the LGB.

Genetic diversity in laboratory samples was generally lower than that of field samples. Similarly, the Malawi population showed a much lower level of mitochondrial genetic diversity than its parental Benin population. This suggests the disproportionate contribution of a few related females to the genetic pool of these populations, population expansion from a few haplotypes, or problems with genetic bottlenecks during recovery, quarantine or rearing (Lloyd *et al*., 2005; Zayed *et al*., 2007). The demographic history of the laboratory populations used in this study is not clear on a few important events. For instance, insects isolated from Mexico were studied in the Benin laboratory but their possible contribution to the Benin populations is not clear (W. Meikle, personal Communication). In Kenya, mite infestation and possible mycoplasma infections were reported leading to culling of a part of the colony, though the eventual founding populations are not documented or the extent of this possible bottleneck known (R. Hodges, P. Likhayo, G. Kibata personal Communication). Recent population genetic shifts and admixture events could have contributed significantly to the population genetics of the populations we used and eventual performance of populations released for biological control. These hypotheses may be tested more efficiently in recent population time scales using hypervariable markers such as microsatellites. The next section of the study therefore focuses on the development testing and use of microsatellite makers to test these hypotheses.

5.6 References

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CHAPTER SIX

Development of Microsatellite Markers for *Teretrius nigrescens*

6.1 Abstract

Microsatellites are the most commonly used neutral multilocus molecular markers in ecological genetics today. Several attributes make them useful in fine-scale variation studies: they are hypervariable, co dominant, reproducible and amenable to automation. Microsatellites were the best markers we could use in addressing the questions raised in the previous chapters which gene sequence data could not answer. However, since none had been developed for Histeridae, fresh markers had to be developed for this study. Twenty four novel microsatellite markers were isolated and characterised. Their population parameters were tested on 50 individuals from a field population from Honduras. Alleles per locus ranged between 2 and 12, and observed heterozygosity between 0.037 and 0.652. Six loci deviated significantly from Hardy–Weinberg equilibrium and showed evidence of null alleles. These markers will be useful for studies of the predator's population structure and characterizing populations for control of LGB.

Published as: **Omondi, A. B., Orantes, L. C., van den Berg, J. Masiga, D.** and **Schulthess, F. (2009).** Isolation and Characterization of microsatellite markers from *Teretrius nigrescens* Lewis (Coleoptera: Histeridae), predator of the storage pest *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) *Molecular Ecology Resources* **9**: 1236 - 1239

6.2 Introduction

Molecular markers are useful in determining the level of genetic differentiation, genetic and demographic history of animal populations. A combination of the molecular markers of appropriate discriminative power and ecological factors may be used to clarify varying levels and possible causes of population fragmentation, differentiation and specialization events. Microsatellites are among the most variable and popular marker systems in genetics. As opposed to unique DNA, microsatellite variation derives mainly from length variability rather than primary sequence variation, and the genetic variation is characterized by high heterozygosity and the presence of multiple alleles hence a high genetic information content. They are used in genetic research for diversity studies, genetic map development, linkage analyses, marker-assisted selection and fingerprinting studies (Ellegren, 2004). There are generally two types of microsatellites those linked to expressed sequences (type 1) and the anonymous ones (type II) associated with non-coding DNA. The EST linked markers are usually more conserved and are useful in selection, breeding and gene mapping studies. Anonymous microsatellites which, are often highly variable, are useful in fine scale population studies of recent genetic and demographic history of populations (Ellegren, 2004; Ng *et al.*, 2005; Vasemagi *et al.*, 2005; Maneeruttanarungroj *et al.*, 2006).

The wide usage of microsatellite markers is based on their co-dominant nature, abundance in eukaryotic genomes, robustness, hypervariability, high information content, requirement for simple sample preparation and amenability to automation (Zane *et al.*, 2002; Selkoe and Toonen, 2006). However, they also have some technical limitations such as the cost of isolation and *de novo* characterization and the number of markers required since one assay forms only one datum point per genotype (Masi *et al.*, 2003). Practical problems also arise in their analysis as many theoretical models fail to accurately explain allele frequency distributions in natural populations (Dakin and Avise, 2004; Ellegren, 2004).

There are several approaches to isolating microsatellite markers depending on the availability of prior information the material and resources available. Several authors have reviewed these procedures, the preliminary evaluation and inclusion criteria for the use of microsatellites in ecological studies (Zane *et al.*, 2002; Selkoe and Toonen, 2006). For widely sequenced species, sequences in genomic databases can be searched for microsatellite repeats and primers designed for these loci. Microsatellites from a closely related species may be used tested and used in a related species with considerable level of success in confamilial organisms (Selkoe and Toonen, 2006). However, the success of the above methods depends on the level of scientific interest in the related taxa. *De novo* isolation may be carried out by developing microsatellite libraries especially for species with little available sequence information.

At the beginning of this work, only 924 Expressed Sequence Tagged (EST) sequences were available from the family Histeridae (Caterino and Vogler, 2002). Microsatellites were therefore developed *de novo* for the *T. nigrescens* to complement information obtained by use of RAPD-PCR, ITS and mtCOI sequence analysis. The objective of this study, therefore, was to develop a panel of novel ecologically useful microsatellite markers for from *T. nigrescens* to test their population genetic characteristics; to test their multiplexing possibilities and assess their potential usefulness in related species.

6.3 Materials and Methods

6.3.1 Microsatellites-enriched library construction

Three microsatellite libraries were developed following the enrichment capture protocol Glenn and Schable (2005). Genomic DNA was extracted from the heads and thoraxes of a pool of ten freshly killed *T. nigrescens* adults of both sexes from the Benin Laboratory population using the Phenol-Chloroform-isolated procedure (Sambrook *et al.*, 1989). About 2-4 µg of total genomic DNA was digested to completion with *Rsa*I, *Bst*U or *Eco*RV and *Xmn*I in (New England Biolabs) in 20 µl volumes. Double stranded linkers (SNX 24 Forward: 5′ GTTTAAGGCCTAGCTAGCAGAATC; SuperSNX24+4P Reverse: 5′ GATTCTGCTAGCTAGGCCTTAAACAAAA) (Glenn and Schable, 2005) were ligated to both ends of DNA fragments in 30µl reaction volumes containing 7.0 µl dsSuperSNX-24 linkers, 1.0 X ligase buffer and 2.0µl DNA ligase and 20 µl of the digested product. Linker-ligated DNA was amplified in 50 µl reaction volumes containing 1X PCR Buffer (with 1.5mM $MgCl_2$); 200 µM dNTPs and 2 Units of *Taq* DNA polymerase (GenScript), approximately 10ng ligated DNA and 200 nM of the Super SNX primer. Thermal cycling conditions were 4 min at 94 C, followed by 30 cycles of 94 °C for 25 s, 55 °C for 1 min, 72 °C for 3 min and a final extension of 72 °C for 15 min. The Super SNX24 forward primer was used as the sole primer.

The PCR products were hybridised to a combination of dinucleotide, trinucleotide and tetranucleotide probes in 50 µl mixtures containing 1X hybridisation solution, 0.2 µM of each of the oligonucleotide probes, 10 µl linker ligated DNA from pervious step. The hybridisation programme denatured the DNA at 95 °C for 5 min then cooled to 70 °C – 50 °C at 0.2 °C every 5 s (99 cycles); 50 °C for 10 m; 50 - 40 °C every at 0.5 °C every 5 s and a final hold at 10 °C. This complex was added to washed and resuspended streptavidin-coated magnetic beads (Dynabead M-28, Invitrogen) and mixed by gentle rotation for 35 min. The mixture was washed twice each with 2X SSC, 0.1 % SDS solution and 1X SSC, 0.1 % SDS at a maximum of 50 °C, each time, capturing the beads with a magnetic particle concentrator (Invitrogen). The enriched fragments were then eluted by denaturing 95 °C for five minutes in TE buffer (10 mM Tris pH 8, 0.2 mM EDTA) and precipitating in 2 volumes absolute ethanol and 0.1 volume NaOAc/EDTA at -20 °C overnight. The DNA was pelleted and washed with 70 % ethanol.

The recovered DNA was double-enriched for microsatellite-containing fragments by nested PCR, in three 25 µl reactions each containing 1X PCR buffer (GenScript), 1.5 mM $MgCl_2$ 200 µM of each dNTP 0.5 µM SuperSNX24 forward primer, 1U *Taq* polymerase (GenScript) and 2 µl of enriched genomic DNA fragments. Thermal cycling was done in Palmer-Cetus (PTC 100) thermocycler at 95 °C for 2 min, 25 cycles of 95 °C for 20s, 60s for 20 s and 72 °C for 90 s and a final elongation at 72 °C for 30 min. Products from the three replicates were bulked for use as templates for the nested PCR. The subsequent PCR fragments were cleaned using MinElute PCR Purification Kit (QUIAGEN) for coning.

The recovered SSR-DNA fragments was ligated into a pGEMT-Easy (Promega) cloning vector according to suppliers' recommendations and used to transform chemically competent *E. coli* (DH5α strain) cells. Transformed white colonies (n = 500) were selected visually from Luria-Bertani/Agar/ampicillin plates with X-Gal and IPTG and screened for the insert by PCR using Super SNX forward primer alone as forward and reverse primer as described in Chapter 2. Positive colonies were therefore those that showed a single band. Two hundred cloned fragments of over 400 bp were sequenced under BigDye terminator cycle sequencing chemistry using M13F-pUC(-40) forward and reverse primers (Macrogen Inc, Korea)

(Chapter 2). Sequences were cleaned to remove vector sequences using Vec Screen and by manual alignment and editing.

6.3.2 Identification of tandem repeats

Microsattellite repeat regions were identified using Tandem Repeats Finder Server (Benson, 1999). This programme allows the finding of SSR repeats without *a priori* specification of the repeat motif or size. It uses statistically-based recognition criteria to identify microsatellites, modelling them according to the percentage identity and frequency of indels. MICROFAMILY software (Meglécz *et al*, 2007) was used to screen sequences for flanking sequence similarity and to eliminate redundant and repeated members of microsatellite families (based on the degree of flanking sequence similarity).

6.3.3 Primer design and optimisation

Flanking sequences were manually demarcated for primer design using an online version of PRIMER 3 software Rozen and Skaletsky (1998). For each locus, forward and reverse primers were designed to anneal at least 20 bp away from the microsatellite core, have at least 30 % GC content and an annealing temperature of about 50 °C. The difference in the annealing temperatures of the forward and reverse primers was set to be within 2 °C of each other. Amplification product sizes were targeted for between 130 and 450 bp to enable allele sizing using a single internal size standard.

Up to six primer sets were selected depending on the characteristics of the flanking sequence, yielding a total of 121 primer combinations (MWG Biotech, Germany). All possible combinations forward and reverse primer pairs were tested for each locus. The initial optimisation reactions contained 1x Genscript *Taq* polymerase buffer with 1.5 mM

$MgCl_2$, 400 µM dNTPs, 150 nM Primers, 1U *Taq* polymerase and ~ 20ng template DNA. The annealing temperature was initially set at 5 °C below the lower melting temperature (Tm) of the primer pair. Where there was no amplification, the annealing temperature was lowered to a maximum of 10 °C below the Primer melting temperature. The highest possible annealing temperature (T_a) at which a definite reproducible amplification product of around the correct predicted size was adopted as the working T_a for that primer set. Primers were screened on two insects from both sexes per population to identify those producing clear reproducible polymorphic amplification product. PCR products were separated in 2 % agarose (in TAE) gels at 2.6 V/cm for 2 hours.

Twenty-seven primer sets that gave amplifications in at least one individual from each population during the pre-screening were selected. For each locus, only one primer set was chosen. Fluorescent dye-labelled primer pairs were designed for these loci for automated multiple genotyping using ABI sequencer (ABI 3130) (Missiaggia and Grattapaglia, 2006). For each primer set, the forward primer was labelled using one fluorescent dye (PET, NED, 6-FAM or VIC) while the reverse primer was not labelled. The PCR products from each co-loading set were diluted and mixed according to the optimum predetermined signal strength. Each analysis well was loaded with 10 µl of sample. Each sample was a mixture of 9 µl of Hi-Di Formamide, (with 0.15 µl of LIZ 500 internal size standard) and 1 µl of the mix of the diluted PCR products. Each co loading set comprised of four PCR products each labelled with a different fluorescent dye. Allele sizing was done in an ABI 3130 or 3730 instrument (Applied BioSystems). Polymorphism and population genetics characteristics were assessed on 50 individuals, randomly selected from wild caught adults, sampled using funnel traps baited with LGB pheromone mimic (Pherocon Technologies) and heat sterilized LGB frass and maize grains. Samples had been collected from Honduras between January and July

2008 and sent to our laboratory alive. This geographic population represented the most recent field recovery.

6.3.4 Allele calling

The sizes of alleles were estimated from electrophoregrams using GENEMAPPER 4.0 software (ABI Biosystems), using LIZ 500 internal standard profile to estimate the allele sizes. The allele detection threshold was set at 200 and 100 for homozygotes and heterozygotes respectively. Peaks were manually edited to confirm correct allele calls. All products that showed equivocal, missing or more than two alleles per sample were assumed unsuccessful and repeated (Figure 6.1). Reactions that did not amplify after two attempts were reamplified with the other sets of earlier primers designed for the locus and the mtCOI primers. Those that amplified were considered null or non-amplifying alleles. A subset of successfully genotyped individuals was re-analysed to test the genotyping error rate of each marker and as positive controls in repeat reactions.

6.3.5 Cross species amplification

Cross species amplification was tested on six histerid species from varying 18S sequence-based relative phylogenetic distance from *T. nigrescens* (Caterino and Vogler, 2002) (Chapter 2). One to six individuals from the species *T. latebricola* (= *T. americanus*), *Chatabraeus* spp. and *Acritus nigricornis* (Abraeinae); *Carcinops pumilio* (Dendrophilinae); *Saprinus bicoloroides* (Saprininae) and *Hister zulu* (Histerinae). The PCR was carried out under the same conditions as those for *T. nigrescens* with two positive controls. PCR products were analysed by direct allele sizing and scored as described for *T. nigrescens* above.

6.3.6 Multiplexing Microsatellite primers

Multiplexing is the amplification of different fragments on a single template, each with its own set of primers, in the same reaction. Products are then separated by size or other attribute on a suitable matrix. Multiplexing microsatellite fragment analysis based on different fluorescent labels reduces the cost and time of the process. However, it involves a potentially erroneous step of coloading set mixing and dilution before analysis. PCR multiplexing reduces the cost of consumables, time and number of reactions necessary and eliminates the need for mixing step. Theoretically, the main considerations of the multiplexing process are primer annealing temperature and dye label. The practical challenges include possibilities of product competition, higher risk of large allele drop out (van Oosterhout *et al.*, 2004), or other causes of failure of generation of specific products. These can lead to problems from false null alleles to inaccurate genotyping and PCR failure. Therefore PCR multiplexing must be optimised for their ability at correct genotyping abilities before use.

Seven possible multiplex sets each comprising three or four differently labeled primers of nearly similar annealing temperatures were tested. Primers quantities were initially set depending on the annealing temperature relative to the others, primer relative amplification and genotyping strength and colour dye (table 3). The PCR assays were carried out as above in 20 µl reaction mixes with 400 µM dNTPs, 2 U *Taq* polymerase and 2 µl template DNA. Amplification with standard *Taq* polymerase (Genscript) and Hot Start (Amplitaq Gold) polymerases were compared. Touchdown PCR was eventually with Annealing Temperature (Ta) decreasing at 0.5 °C per cycle (from highest Ta + 1 degree to lowest Ta from earlier optimization reactions). Products were loaded undiluted (0.5 µl + 10 µl) of Hi-Di formamide containing 0.12 µl of LIZ 500 internal size standard.

6.3.7 Data Analysis

Allele frequencies and deviations from HWE were calculated with Bonferroni corrections using CERVUS 3.0 (Kalinowski *et al.*, 2007). The occurrence of null alleles for each marker was estimated with MICROCHECKER at 95 % level of significance with 1000 iterations (van Oosterhout *et al.*, 2004). Linkage disequilibrium was tested using GENEPOP version 3.4 software (Raymond and Rousset, 1995; 2004), under both the Fischer's and Markov Chain method (10000 dememorization steps, 100 batches and 1000 iterations per batch). For this test, only samples collected from Tegucigalpa, Honduras, were included to avoid the possible effect of the genetic structure and covariation of alleles between populations. Marker reliability was assessed by re-amplification of 10 randomly selected individuals twice and comparing the results.

6.4 Results

Of the 200 fragments sequenced, 64 fragments with microsatellite repeats were screened for flanking region similarity using MICROFAMILY (Meglécz *et al.*, 2007) (Table 6.1). About three primer pairs per locus were designed using PRIMER 3 (Rozen and Skaletsky, 1998) for the resulting 39 unique fragments. Twenty-seven primer pairs consistently amplifying loci from both sexes and six test populations were selected for polymorphism assessment. The loci TnM58, TnN6 and TnN13 gave inconsistent amplification while TnM50 ([GGTAGAA]$_7$) produced uncorrectable stutter peaks on while the markers was clearly polymorphic on agarose gel analysis (Figure 6.2). General amplification failure was observed with TnM541 and TnN12. The number of alleles per locus ranged between two and 12 (average = 5.75) (Table 6.4). Seven loci deviated significantly from Hardy Weinberg Equilibrium (Table 6.4). There was also evidence of presence of alleles in all these loci and in TnM541 and TnN21 (Table 6.4). Three groups of markers showed significant linkage disequilibrium: TnM36-TnM70-TnM53; TnM79-TnM85 and TnM41-Tnm71. A BLAST query of available sequences in the public genetic database did not reveal any significantly similar sequences.

Cross amplification was successful for most samples and most loci (Figure 6.3, Table 6.5). About 80 % of all the primers selected for *T. nigrescens* amplified the other Abraeinae specimens and 70 % were polymorphic. Histerinae (*H. zulu*) and Dendrophilinae (*C. pumilio*) gave the poorest amplification and polymorphism under the conditions used for *T. nigrescens*.

6.4.1 Multiplex sets

Multiplexing sets B, C, D, E and F produced correct genotyping reactions for the four multiplexing test samples with both standard (GenScript) and Hot Start polymerases (Amplitaq Gold; Applied Biosystems), after a single trial (Figure 6.4). The reactions that failed in these multiplexing sets had also failed in the simplex reactions. Multiplex set ‘A’ was the least successful, with only half the reactions being unequivocally scorable.

Table 6.1: Marker attrition rate in the development of *T. nigrescens* microsatellites

Parameter	Number	%of last	% of original
Number of positive clones	324	-	100.00
Clones with >400bp (sequenced)	200	61.73	61.73
Sequences with SSRs	64	32.00	19.75
Non-redundant SSR sequences	39	60.94	12.04
Amplified primer sets (each population)	66	51.97*	-
Amplified loci	33	50	20.37
Primers selected for genotyping	27	81.82	8.33
Polymorphic loci	24	88.89	7.41

* Percentage of the 127 primer sets developed. For each locus, at between two and six primer sets targeting regions of different sizes were designed and tested.

Table 6.2: Frequency of various types of repeats in sequenced motifs

Repeat Type	Isolated	Amplifying	Remarks
Mono-	11	-	**Associated with other repeats**
Di-	18	9	Most very short or not amplifying
Tri-	48	17	Good candidates
Tetra-	3	1	Long (CATA)n candidate not amplifying
Penta-	1	0	Not amplified
Hexa-	4	0	Some resolved to trinucleotide repeats
Other	1 (septa-)	1	Variable on gel, many stutter peaks
Compound	26	6	One and >1 repeats together

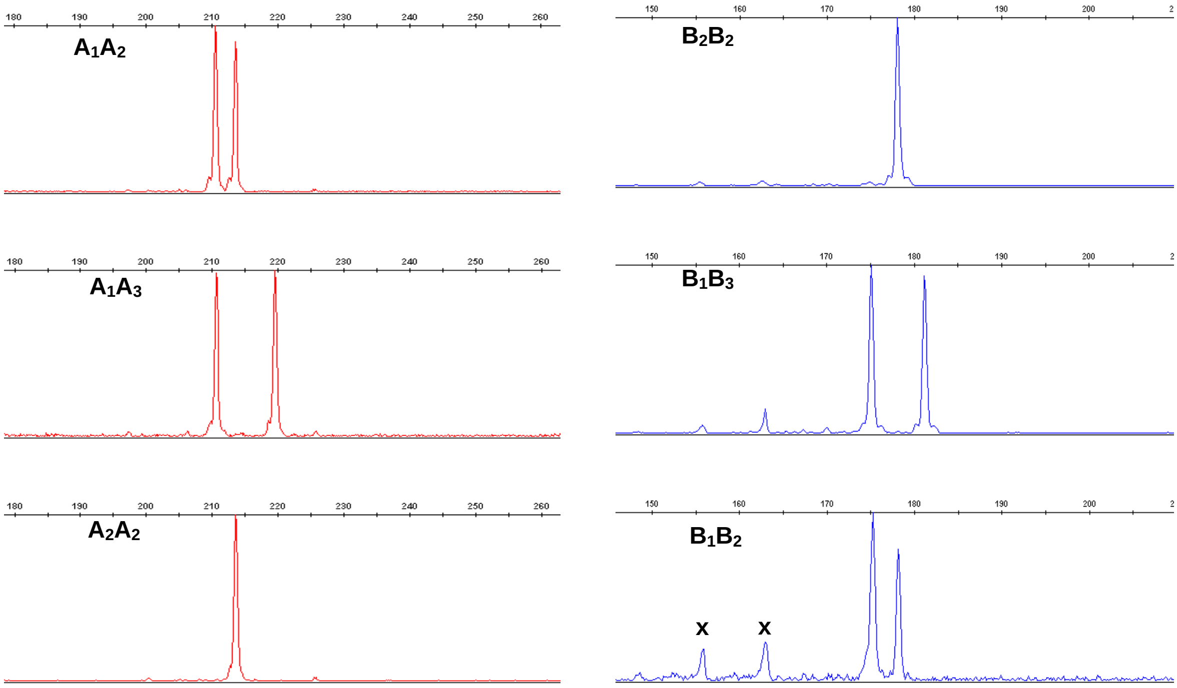

Figure 6.1: Alleles scored by their size in base pairs using GENEMAPPER. The expected lengths are marked in for automatic correction of sizes of all alleles falling within this bin range. Three individuals are scored on two loci and three different alleles. **'x'** represents pull up or background noises visible during genotyping.

Table 6.3: PCR conditions for multiplex sets of primers. Ta refers to the conditions used in the final reactions

Set	Name	Dye	Ta	Primer Qty	Reaction Ta	Success rate
A	TnN1	6-FAM	48	250	**53 – 48**	1/4; 2/4
	TnM70	VIC	49	250		inconsistent
	TnM47	NED	50	350		Non specific
	TnM48	PET	51	350		4/4; 4/4
B	TnM50	VIC	50	375	**55 - 50**	Non specific
	TnM36	6-FAM	51	375		4/4; 4/4
	TnM58	PET	52	500		No amplification
	TnM71	NED	53	500		2/4; 3/4
C	TnM40	VIC	52	375	**55 - 50**	4/4; 4/4
	TnM66	6-FAM	51	375		4/4; 4/4
	TnN28	NED	53	500		4/4; 4/4
	TnN2a	PET	54	500		4/4; 4/4
D	TnM54	PET	53	500	**56 - 52**	4/4; 4/4
	TnM85	VIC	53	500		3/4; 3/4
	TnN12	NED	54	500		Non specific
	TnM79	6-FAM	53	250		4/4; 3/4
E	TnN21	VIC	53	350	**56 - 52**	3/4; 3/4
	TnN13	PET	53	500		Non specific
	TnM23	NED	54	500		3/4; 3/4
	TnM55	6-FAM	53	350		3/4; 3/4
F	TnM541	NED	55	500	**58 – 52.5**	2/4; 2/4
	TnM72	PET	55	500		4/4; 4/4
	TnM53	VIC	56	400		3/4; 2/4
	TnM51	6-FAM	53	350		2/4; not specific
G	TnM41	NED	56	250	**58 – 51**	2/2; 2/2
	TnN6	PET	54	350		No amplification
	TnN14	6-FAM	51	500		2/2; 1/2

For each amplification set, the fraction represents success rate (for production of the correct genotype as simplex reactions) when attempted on the four samples of known genotypes. The first fraction represents GenScript *Taq* polymerase and the second Amplitaq Gold master mix.

Table 6.4: Characteristics of 24 microsatellite loci and primers for *T. nigrescens* tested on 50 LGB pheromone trap-sampled adults from Honduras.

Locus/ Accession No.	**Repeat motif**	**Primer sequence 5′ – 3′**	**Ta (°C)**	**Size range (bp)**	**N**	**n_A**	***Ho***	***H_E***
TnM47 EU826153	$(CAT)_7$	**F: NED-**AAATCGCTTTTCTTCTTGCAT **R:** ATGCACGTGATTTTGCATTC	50 [A]	140 - 169	38	5	0.053	0.366†*
TnM48 EU826156	$(ATT)_3(ATG)_6GGG(ATG)_{10}$	**F: PET-** CCCTTTGACGGCATTGTTTA **R:** TGCGTTCTTGAAATGCAAAC	51 [A]	211 - 220	42	5	0.286	0.546†*
TnM55 EU826152	$(CCG)_5\cdots(ATG)_6$	**F: 6-FAM-**GGCCCCAACTTTCCATTATT **R:** CTTTACAGGTCCCCGCTTTC	53[E]	172 - 184	39	6	0.118	0.168
TnM70 EU826157	$(TGA)_{16}(AAC)_{10}$	**F: VIC-** GAAGAATTCTACTAATTTATATGTG **R:** GGGTGAGCAATATTTTAATG	49 [A]	239 - 252	39	6	0.419	0.400
TnM71 EU826158	$(ACT)_{17}$	**F: NED-**CACGCTGGGTGGTGAATAG **R:** TTCTTTCCCGAAAGCTCAAC	53[B]	208 - 211	47	5	0.511	0.628
TnM53 EU826151	$(CATT)_4..(TG)_{17}$	**F: VIC-** GCCGAGCGCATTAGATACAT **R:** ACAGCTGATGTTCTTCCAACG	56 [F]	167 - 217	36	5	0.417	0.686†*
TnM79 EU826160	$(TGA)_{17}$	**F: 6-FAM-**CTCGATTGCTCATGTGTTCG **R:** TTAGAATGTGGCGGGATTGT	53[D]	183 - 193	47	4	0.128	0.160
TnM85 EU826161	$(TACA)_9$	**F: VIC-** GTTTTGCGGAAAGACCGTAG **R:** TGAGCCGACCAACTTAATGA	53[D]	193 - 239	46	10	0.652	0.683
TnM36 EU826154	$(GAT)_{20}$	**F: 6-FAM**-ACATGTTAAGGATCCGAAAACC **R:** CGATGCTTTTTCATTTGAGC	51[B]	166 - 181	50	4	0.600	0.568
TnM51 EU826155	$(GCT)_7$	**F: 6-FAM-**GCAACAAGATTCCGAACAATC **R:** GTTTCCGTTTGAGTGCGTTT	53[F]	163 - 166	45	3	0.133	0.205
TnM54 EU826149	$(TGA)_6$	**F: PET-** CCAGCTGAAATAGGCACAAA **R:** CGGCACAATATATTCCAAAGAA	53[D]	206 - 217	39	2	0.065	0.104
TnM72 EU826159	$(CAA)_{22}(CAG)_2(CAA)_{29}$	**F: PET-** AGGTCCGACCGTTGGTAAC **R:** GAACGCACCACCGATACTTT	54[F]	194 - 214	45	8	0.622	0.652

Table 6.4 continued

Locus/ Accession No.	**Repeat motif**	**Primer sequence 5′ – 3′**	**Ta (°C)**	**Size range (bp)**	**N**	**n_A**	***Ho***	***H_E***
TnN21 EU826167	$(TCA)_{40}$	**F: VIC-** CACAAAGTCAATTTCGACGTG **R:** AACCACCATTTGACCTGGAA	53[E]	209 - 228	50	5	0.320	0.397†
TnN28 EU826168	$(GAT)_8$	**F: NED-**TAGCGCAGAAGGACAAATGA **R:** CCCTGGGTACATGCAAAAAT	53[C]	175 - 183	37	3	0.060	0.169†*
TnM541 EU826162	$(CA)_6(GAT)_6$	**F: NED-**GATTTCCATCGTCCTGGCTA **R:** CTGACCTGTGTTCGTTTTGC	54[F]	163 - 186	27	2	0.037	0.037†
TnN12 EU826165	$(TGA)_{17}$	**NED-**GGAGAAAAACTGCGAAGTTACG **R:** TCCAATTAGAAAGTGGCTGGA	54[D]	229 - 237	19	4	0.316	0.585†*
TnN14 EU826166	$(CTT)_{11}$	**F: 6-FAM-**AAGACGCCGTTTTCTTGAAT **R:** TAAATTTCCGGTTGCCTTCC	51[G]	133 - 146	48	5	0.146	0.196
TnN2 EU826164	$(TTG)_{60}$	**F: PET**-CTAGGCTTTGTTGTTGTTGC **R:** AGGAACACTCAAGAACATTCG	53[C]	216 - 234	48	4	0.375	0.434
TnM23 FJ531687	$(CA)_{16}(CT)_{12}$	**F: NED**-CGGTCTCTTAAGGACAGAAGTTG **R**: GAGGGAAAATGAATTGACAAGG	54[E]	140 - 166	48	12	0.500	0.811†*
TnN1 FJ531692	$(TTG)_{34}$	**F: 6FAM-**AATTCTAGAGCAGTAGGC **R:** TTCCAACAGACGTTCCAACA	48[A]	253 - 272	48	4	0.396	0.484
TnM40 FJ531688	$(CA)_{13}$	**F: VIC-**CTGCTACGAAACTGGTGCTG **R:** GCCAAAGACGAGCCATAAAT	52[C]	145 - 156	50	6	0.580	0.686
TnM41 FJ531693	$(GAT)_{10}$	**F: NED-**AAGTAACGAGGGCGAGAAGAC **R:** GTAGAATCCGCACCCAGAAG	55[F]	116 - 131	48	6	0.646	0.630
TnM58 FJ531689	$(GT)_{11}$	**F: PET-**CGCAATGGTGTTCCTGTTAG **R:** AAAAACCCGAGAGCCAATTC	52[B]	116 - 131	34	5	0.529	0.652
TnM66 FJ531690	$(GA)_{14}$	**F: 6FAM-** GGAAGGTTCAAGCTGTCCAA **R:** ATGCCTTCCCGTACGATTT	51[C]	141 - 179	46	11	0.196	0.491†*

Interrupted repeat motifs are denoted by (…).
All forward primers were directly labelled on the 5' end, reverse primers were not labelled.
† markers showing existence of null alleles, * loci showing significant deviation from Hardy Weinberg Equilibrium.
Ta: annealing temperature; multiplex sets tested shown by [A, B, C, D, E, F].
N: number of individuals successfully genotyped, n_A: number of alleles detected.

Table 6.5: Cross-species amplification of 19 microsatellite primers isolated from *Teretrius nigrescens* on seven species in four subfamilies within the family Histeridae.

Locus	*Teretrius americanus*	*Acritus nigricornis*	*Abraeus sp*	*Chatabraeus sp*	*Saprinus bicoloroides*	*Carcinops pumilio*	*Hister zulu*
TnM23	+	+	+	-	-	-	-
	148 - 159	144 - 161	161 – 175				
TnM40	+	+	+	-	+	-	-
	141 - 178	144	144		154		
TnN1	+	+	+	-	-	-	-
	263 - 272	263	235 – 263				
TnM66	+	+	+	+	-	-	-
	146 - 150	143	243	262			
TnM36	+	+	+	+	+	+	+
	175 - 181	174 - 178	178 – 181	178 - 181	175 - 178	178 - 181	175 - 178
TnM47	+	+	+	-	+	+	+
	162 - 226	162 - 226	162 – 226		162	162 - 234	162
TnM48	+	+	+	+	+	+	+
	211 - 220	211 - 214	214 – 220	220	211 - 220	211 - 214	214 – 220
TnM55	+	+	+	+	+	-	-
	175 - 179	184	184	184	184		
TnM70	+	+	+	-	+	-	-
	249	254	238 – 254		249 - 254		
TnM71	+	+	+	+	-	-	-
	195 - 208	208	208	208			

Locus	*Teretrius americanus*	*Acritus nigricornis*	*Abraeus sp*	*Chatabraeus sp*	*Saprinus bicoloroides*	*Carcinops pumilio*	*Hister zulu*
TnM51	+	+	+	+	-	+	-
	160 - 163	163	163	163		163	
TnM53	+	+	+	+	+	+	-
	202 - 205	202 - 205	202	202	202	202	
TnM54	+	+	+	+	-	+	-
	195 - 216	203 - 216	203 – 216	214 - 217		216	
TnM541	+	+	+	+	-	-	-
	187	187	187	164 – 217			
TnM79	+	+	+	+	-	-	-
	137 - 146	176 - 192	176 – 198	176 – 198			
TnM85	+	+	+	+	-	-	-
	232 - 237	232	232	232			
TnN12	+	+	+	+	-	-	-
	232 - 237	233	233	233			
TnN2	-	+	+	+	-	-	-
		226	222 – 226	226			
TnN21	+	+	+	+	-	-	+
	222 - 238	222	221	222			212
TnN28	+	+	+	+	+	-	-
	170	170 - 222	170 – 177	170 - 177	177		

Successful amplification of at least one sample (+), non-specific or failed amplification (-); size range of the amplified products (from automated genetic analyzer) is given.

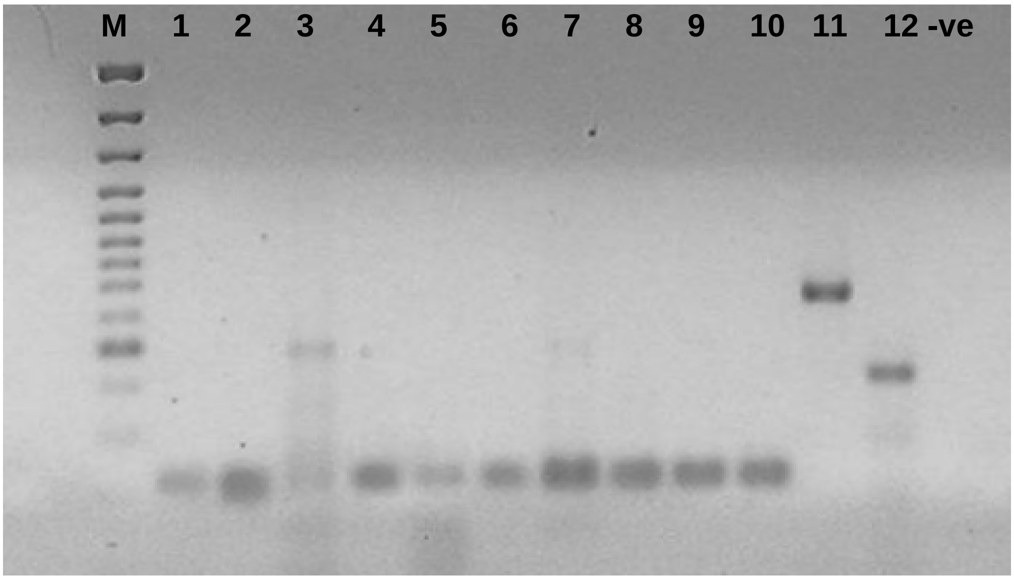

Figure 6.2: PCR products of TnM50 marker on 2 % agarose gel populations. Accessions: M (100 bp DNA size standard); 1-2 (KARI); 3,4 (Benin); 5-6 (Batan); 7-8 (Oaxaca); 9-10 (NRI) 11-12 (Tlaltizaspan) and 13 (-ve control – water).

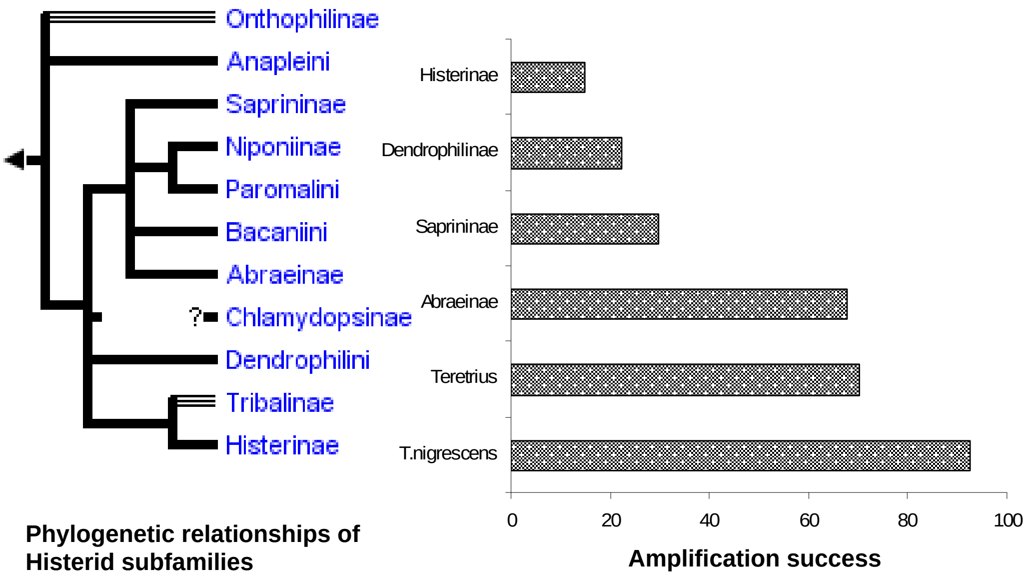

Figure 6.3: Comparison between the phylogenetic relationship of Histeridae subfamilies *sensu* Caterino and Vogler (2000) (left) and percentage cross amplification success of 27 unique microsatellites isolated (right).

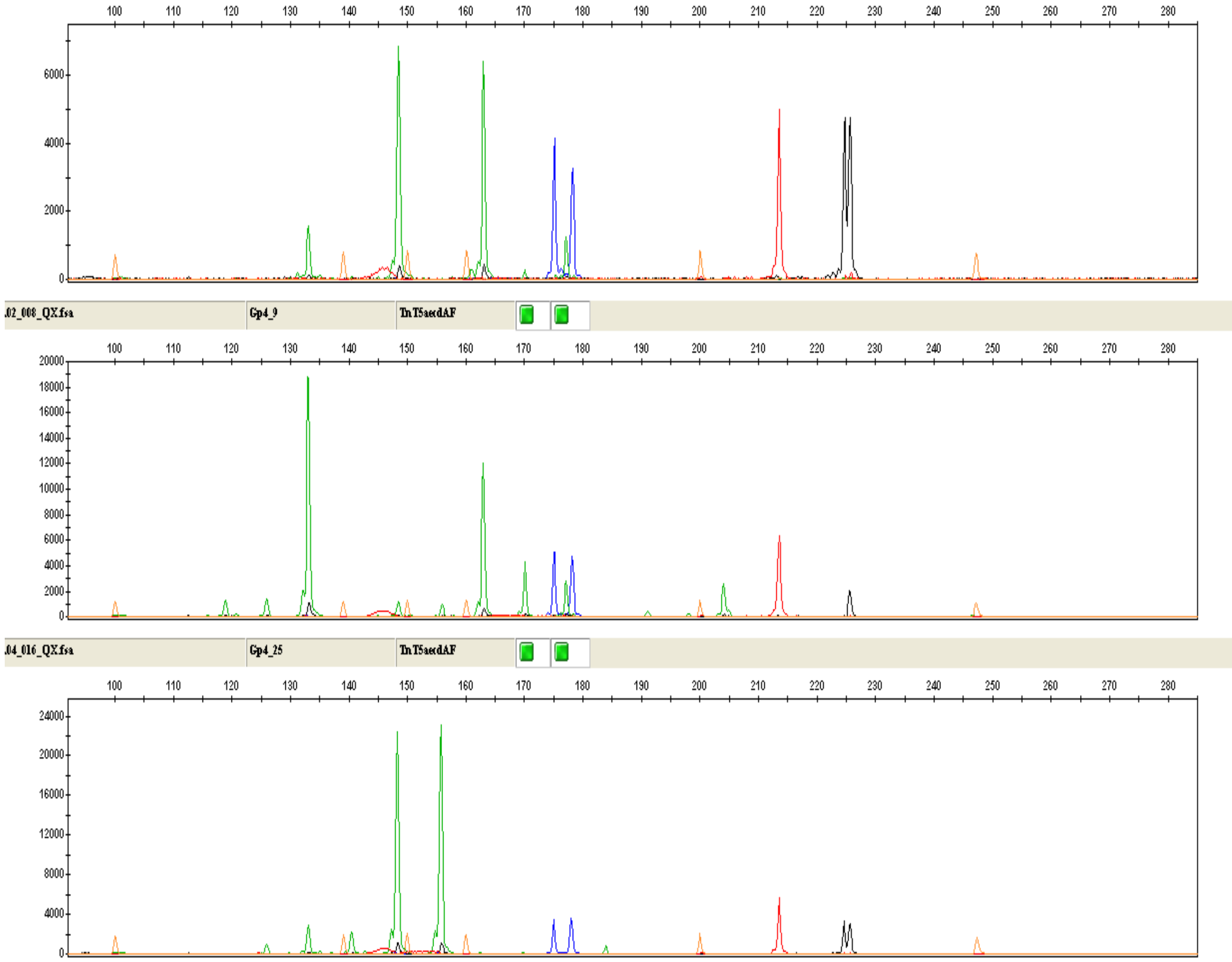

Figure 6.4: Allele sizing profiles from multiplex analysis of amplicons from genotyping reactions. Profiles show a: size standard (orange colour peaks) and products labelled with the four fluorescent dyes. Relative signal strength is shown on the height (x – axis) while y-axis shows the fragment size.

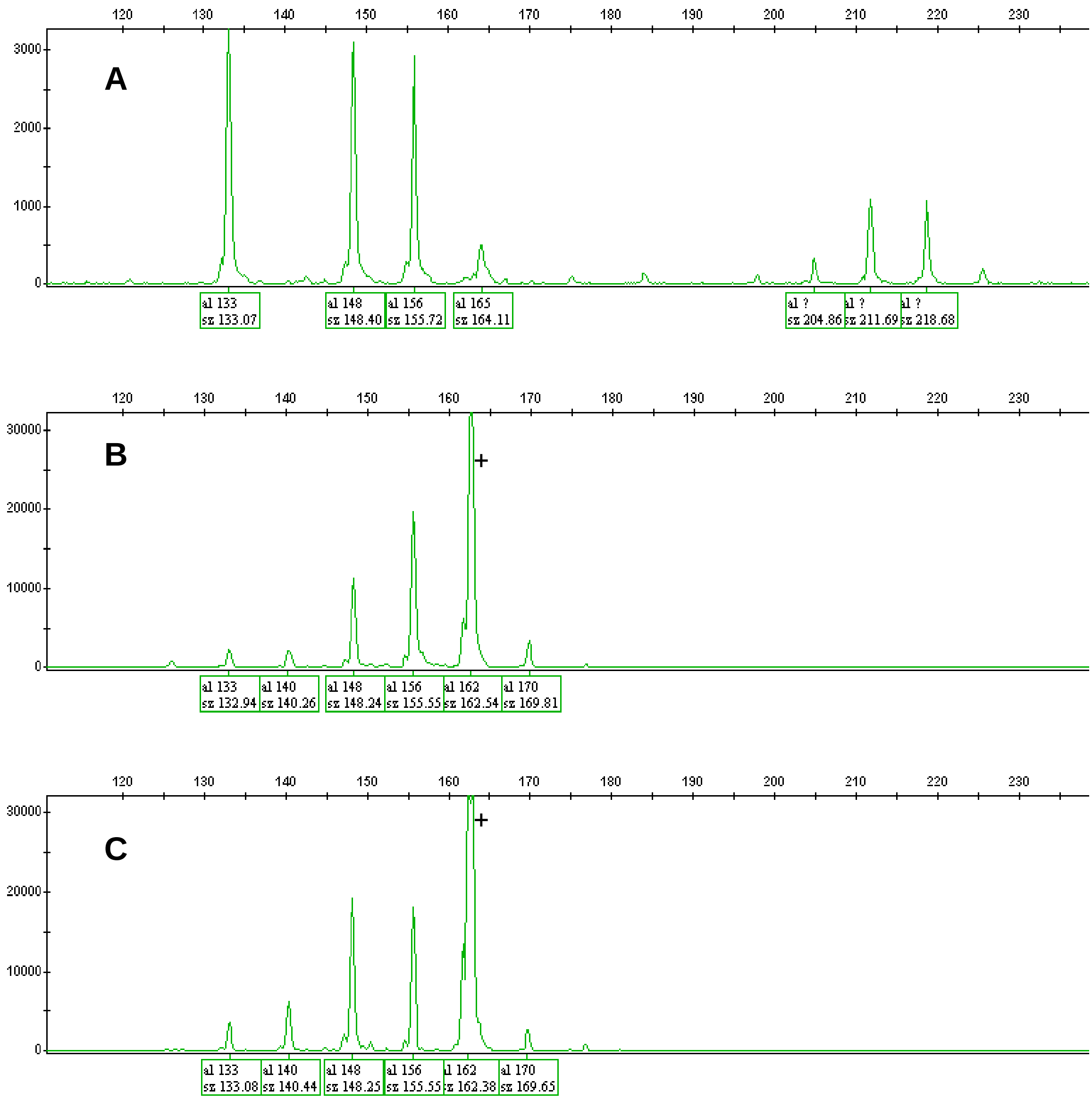

Figure 6.5: Stutter bands associated with TnM 50 from a random sample of three genotypes. A and C are perhaps heterozygotes while B is most likely a homozygote as it displays a clear reduction in stutter peak height away from the main peak. '+' denotes the probable right allele. **A** is also a potential result of amplification of two alleles from the same family from different locations in the genome (Meglécz *et al*., 2004).

6.5 Discussion

Dinucleotide repeats are the most abundant and polymorphic followed by mono and tetranucleotide repeats, while trinucleotide repeats are the least dominant (if a high minimum size threshold is considered) (Fornage *et al.*, 1992; Ellegren, 2004). In fact several studies using a mixture of probes have ended up either solely or mostly with dinucleotide repeats (Theissinger *et al.*, 2008). Since they are also the most variable it is also common that microsatellite isolation studies focus solely on these motif types (Khamis *et al.*, 2008; Vinson *et al.*, 2008). In this study, trinucleotide repeats were the most abundant accounting for (62 %) of all SSRs isolated and (71 %) of eventual SSRs genotyped. It is not clear why this occurred. For a given repeat length, dinucleotide repeats would be 33 % shorter than the trinucleotide repeats, hence would elute at a lower temperature during the washing steps of hybridisation capture. A second library construction using dinucleotide probes alone yielded only an additional 3 microsatellites in the final genotyping. As expected, dinucleotide and tetranucleotide repeats were generally more variable (average 9.7 alleles per locus) compared to trinucleotide repeats (4.94 alleles per locus). The marker attrition rate was higher among dinucleotide repeats where 66 % of those isolated did not eventually meet the utility criteria (Selkoe and Toonen, 2006). Although 87 % of them were unique, most failed to give reproducible amplifications, were not in HWE or showed significant evidence of null alleles. All these observations were possibly as a result of hypervariability of both the core repeat motifs and flanking sequences of these markers, which made universal primer annealing sites difficult to find.

Accurate microsatellite scoring is often complicated by the presence of stutter bands. These bands introduce possible error in the genotyping of populations if not accommodated (Dakin and Avise, 2004). Stutters are inaccurate peaks caused by the introduction of errors during

PCR, due to polymerase slippage and the amplification of the incorrect copies amplifying products that are multiples of repeat motif longer or shorter than the template allele (Lai and Sun, 2004). Their occurrence in earlier cycles leads to accumulation that masks the correct allele sizes, making it difficult especially to differentiate homozygotes from heterozygotes. Such errors are more likely in mononucleotide and dinucleotide repeats, but are also found in other peaks (Walsh *et al.*, 1996). Clarke *et al.* (2001) found high error rates in TA and CA repeats longer than 18 repeat units, with both *Pfu* and *Taq* polymerases. Several microsatellite development assays drop markers showing significant levels of stutter repeats (Selkoe and Toonen, 2006). However, stutter repeats can be corrected for clearly scorable peaks by allele scanning programmes if known, this problem is perhaps not quite serious. In this study, one marker (TnM50 $[GGTAGAA]_7$) showed great variability on gel but abundance of stutters that differed by 7 bp (Figure 6.5). It was impossible to score this marker meaningfully, partly due to the high stutter ratio and absence of a provision for a heptanucleotide repeat in the microsatellite repeat scoring and scanning programmes available.

Multiplexing was done at the allele sizing level (using colour dyes). Multiplexing enables the simultaneous analysis of several markers in a single reaction hence reduces the cost of analysis and increases genotyping efficiency. This also reduces the ardour of PCR analysis, promoting automation and reducing potential error and contamination. PCR multiplexing, amplifying several markers in a single reaction further enables simultaneous analysis of several markers. However, PCR multiplexing requires careful optimisation to determine which samples can be run together to ensure comparable ranges of signal strength and reduce allele drop out or amplification failure due to preferential amplification of certain fragments (Masi *et al.*, 2003). Some loci earlier initially failed to amplify multiplex sets when primers were used in equimolar concentrations, especially with rather difficult loci. Adjusting the relative primer concentrations relative to their signal strength in simplex reactions and observed peak dominance (Table 6.5).The multiplex analysis used here can be expanded to include primers with similar colour labels and non-overlapping size ranges once they can be convincingly established in various populations. While multiplexing provides a cost effective option to microsatellite assays, its use with these markers must be preceded by careful optimisation for template quality, polymerase, primers and laboratory conditions, since it is not clear they might be easily repeatable across laboratories.

Eight loci deviated significantly from Hardy Weinberg Equilibrium in the wild Honduras population used for maker characterization (Table 6.4). This could have been caused by the Wahlund effect or existence of genetic substructure given that the Honduras population was sampled three times across six months period and a geographical range of about 400 km and > 1500 altitude difference. There was also evidence of null alleles in all these loci and in TnM541 and TnN21. Other than mutations in the microsatellite annealing sites, the markers showing null alleles could also have been associated with coding DNA (within or physically linked to exons) which if under selection favouring one variant, could lead to the disproportionate survival of individuals bearing this. This and the distribution of null alleles may explain why some loci alone were in significant deviation from HWE.

Three groups of markers showed significant gametic disequilibrium ($p < 0.01$): TnM36-TnM70-TnM53; TnM49-TnM85 and TnM41-TnM71. In the MICROFAMILY analysis, that compared by sequence alignment each set of flanking sequence to a database containing all the microsatellite sequences identified, all these primers were listed as unique. Co-variation between these loci could have been caused by the physical proximity of these loci on the chromosome allowing joint inheritance of the alleles. Only a single member of each group would thus be useful for population analysis if neutral markers are needed.

Cross species amplification is possible because of the highly conserved nature of microsatellite flanking sequences enabling their use in studies of related species, often to the family level (Ottowell *et al.*, 2005; Selkoe and Toonen, 2006). However, the level of polymorphism and success of amplification declines with an increase in the phylogenetic distance between the source and test species (Primmer *et al.*, 1996; Neff and Gross, 2001; Wright, 2004). Most of the primers in this study isolated were able to amplify loci of other species in the family Histeridae confirming the observations in plants and vertebrate taxa (Peakall *et al.*, 1998). The success rate declined by the 28S r-DNA sequence based phylogenetic distance between the test species and *T. nigrescens*, based on the phylogenetic distance between histerid subfamilies (Caterino and Vogler, 2002). Curiously, TnN2 failed to amplify in the congeneric *T. americanus*, yet amplified in the other Abraeinae and distantly related Histerini. Although some decline in allelic diversity was observed in this study, statistical ascertainment of this bias was not done owing to the dearth of individuals of related species. Such a study would need genotyping at least 20 well-sampled individuals of at least one wild population of these species, which was beyond the scope of this study. The success rate observed with these loci however shows that these primers good candidates in the similar analyses and microsatellites marker development in the family Histeridae, especially Abraeinae, Saprininae and related subfamilies. The success rate and reliability of these primers may be increased by further optimization, relaxing annealing stringency and increasing DNA quantity in the PCR mix, which we did not do. Being the first set of primers developed in this family of beetles and due to the paucity of histerid sequences in genetic databases, these markers will be useful in genetic studies in this family.

The criteria for identifying and testing microsatellites to be used in ecological studies (Selkoe and Toonen, 2006) and techniques of overcoming some practical problems with microsatellite analyses (Dakin and Avise, 2004) improve the accuracy of data generated from this technique. Hardy Weinberg Equilibrium, Mendelian inheritance, repeatability and scorability have already been tested in the 24 loci isolated in this study. These will be used for further testing and evaluation of these loci for use in the population studies of *T. nigrescens.*

This study successfully developed and characterisaed the first set of microsatellite markers for *Teretrius nigrescens*. To our knowledge, this is also the first set of SSR markers developed in the family Histeridae. The markers cover a range of repeat types, and levels of variability, and should find utility in ecological and genetic studies of this and related species. The next chapter describes the use of these tools in the studies of genetic structure and demographic history of 13 geographical populations of *T. nigrescens.*

6.6 References

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CHAPTER SEVEN

Population Genetic Structure and Demographic History of *Teretrius nigrescens*

7.1 Abstract

Molecular markers have so far, in this work shown the existence of two distinct maternal genealogies of *T. nigrescens*. It is possible that genetic and ecological interactions accompany this differentiation, but this has not been clear. This study uses the hypervariable markers developed earlier in the work to study the genetic population structure, eco-genetic differentiation and demographic history 13 sample populations of *T. nigrescens*. A total of 432 individuals from the putative populations were genotyped at 21 polymorphic microsatellite loci. The data were analysed with standard statistical methods and Bayesian approaches. The loci were polymorphic for most populations. Genetic diversity was higher in field than laboratory populations. Population bottlenecks were not detected, but recent expansion was observed in laboratory populations. The samples separated into five informative groups, bringing together populations from the same geographical zones. However, population structuring was hierarchical to a maximum of nine populations. While geographical distance had contributed to population genetic identities, the introduction of the predator into Africa resulted in significant genetic drift and allelic equilibrium changes. Thus, a genetic quality control approach to biological control is suggested.

7.2 Introduction

Hypervariable molecular markers are potentially essential tools in biological control. They can be used for the detection of significant historical demographic events, genetic variation and monitoring of established populations (Lowe *et al*., 2004; Lloyd *et al*., 2005). Classical biological control is the introduction of a co-evolved natural enemy meant to control an inadvertently introduced invasive pest. Both introductions are often followed by rapid population expansion in the new geographical range usually leading to founder events depending on the size of the founding population (Grevstad, 1999; Sakai *et al*., 2001; Memmott *et al*., 2005). If the introduced populations are small, a significant reduction in allelic diversity through genetic drift and inbreeding occurs (Nei *et al*., 1975). For natural enemies, this effect can be reduced by large releases, multiple isolation from the field over the expanse of the genetic range (Nei *et al*., 1975; Grevstad, 1999; Roderick and Navajas, 2003; Lloyd *et al*., 2005). However, initial population sizes may not be informative without knowledge of the underlying natural diversity, which could be exploited to increase diversity (Lockwood *et al*., 2005; Bacigalupe, 2008). Also, the initial diversity can easily be lost in laboratory culturing if strong selection forces occur to enable the organism to survive under such conditions (Bigler, 1992). Such may result in lower vigour of the natural enemy in the wild (Bellows *et al*., 1999).

Nuclear microsatellite markers are highly variable and informative in population genetics. They are distributed throughout the eukaryotic genome and are amenable to genetic recombination through mating, hence useful in the studies of population structure, demographic dynamics, population movements and dispersal over variable time scales (Beaumont *et al*, 2001, Liu *et al*., 2009, Zhang and Hewitt, 2003; Ellegren, 2004; Bray *et al*., 2009). In classical biological control, they can be used to investigate the source and area of origin of an introduced pest population, population-specific monitoring of changes in genetic diversity and the relationship between ecological suitability and genetic diversity. For instance, Lloyd *et al*. (2005) used microsatellite and mitochondrial markers to examine the effects of collecting, quarantining and establishment of the spurge gall midge *Spurgia* *capitigena* (Bremi) (Diptera: Cecidomyiidae), a natural enemy of *Euphorbia esula* (L.) on its genetic diversity. Nuclear microsatellite markers have also been used in the study of invasion genetics (Liu *et al*., 2009), conservation biology (Beaumont *et al*., 2001) and even in human population genetics (Bonhomme *et al*., 2008).

Teretrius nigrescens was introduced into Africa principally as two geographic populations - one from Costa Rica and one from Mexico- released separately (Giles *et al*., 1996; Meikle *et al*., 2002; Hill *et al*., 2003). A phylogeographic study of the mtCOI sequences of 10 local populations has shown the existence of two distinct mitochondrial lineages of *T. nigrescens* (Chapter 6) with possible coexistence of the mitochondrial halpotypes in mid-altitude populations of southern Mexico. Mitochondrial genes are generally clonally inherited, hence invaluable in determining population lineages and species boundaries (Adams and Palmer, 2003; Thao *et al*., 2004). However, they may not reveal genetic recombination or recent population genetic events (Loxdale and Lushai, 1998).

Genetic variation possibly had a role in the variability of *T. nigrescens* success in Africa (Anonymous, 1999), although its contribution has not been studied directly. Also, despite the importance of genetic diversity in biological control, little effort is usually given to maintaining diversity in the colonies and the genetic diversity of the released specimens relative to the native populations is virtually unknown. This study sought to provide baseline information on their diversity and ecological suitability is necessary for eventual informed management and eventual monitoring of their establishment in the field. To address these issues, neutral microsatellite markers were used to investigate the fine-scale population structure and demographic histories of the *T. nigrescens* populations from a north-southerly transect between central Mexico and north eastern Costa Rica and of samples from the region already introduced into Africa (Meikle *et al*., 2002).

7.3 Materials and Methods

7.3.1 Sampling and PCR

A total of 432 samples of adult *T. nigrescens* were genotyped. Samples were variously obtained from laboratory and field colonies as described in Chapter 2. Sample preparation and DNA extraction were done using the standard phenol-chloroform protocol (Sambrook *et al*., 1989).

Genotyping was done in simplex PCR reactions as described in Chapter 2 using 21 variable labelled microsatellite primers developed by Omondi *et al*. (2009). Allele sizing was done on an ABI sequencer, with LIZ 500 internal standard. Genotypes were scored using GENEMAPPER Software Version 7 (ABI Biosciences) and exported into MS Excel worksheet through text editing applications for collation and preparation of input files. Any samples that failed to amplify were reanalyzed three times in simple PCR. Individuals that consistently failed to amplify with microsatellite markers were amplified with a 1300 bp fragment of the mtCOI gene and the ones that failed were excluded. Most of these individuals had a traceable handling history pointing to poor DNA quality.

7.3.2 Data Analysis

Null alleles occur when some samples fail to amplify with certain markers perhaps due to a mutation within the primer annealing site. Such mutations prevent annealing hence PCR does not occur under optimized conditions. Such results may be discerned from a general lack of amplification due to inadequate DNA quality; as such individuals amplify well at other loci. All non-amplifying samples were therefore re-amplified twice for each failed locus to differentiate null alleles from amplification failure. MICROCHECKER software (van Oosterhout, 2004) was used to evaluate the existence of null alleles for each locus.

Genetic Diversity

The genetic diversity was tested with various applications. CERVUS version 3.0 (Kalinowski *et al*., 2007) was used to calculate the number of alleles (n_A), allele frequency and Hardy Weinberg Equilibrium (HWE) expectations of heterozygosity (H_E and H_O). GENEPOP version 3.4 (Raymond and Rouset, 1995) was used to test for population departure from HWE, and linkage disequilibrium using Markov Chain Monte Carlo (MCMC) methods with 10,000 replications and 10,000 dememorisation steps. The allelic richness (n_R) was determined using FSTAT version 2.9.3.2 (Goudet, 2002). Fixation indices (F_{IS}) were calculated according to Weir and Cockerham (1984) using GENETIX version 4.04 software package (Belkhir *et al*., 2002).

Demographic history

Recent demographic events such as migration, population bottlenecks and expansions leave a genetic signature in the genotypes and allele frequencies of present day populations (Cornuet and Luikart, 1998; Luikart and Cornuet, 1998). Population contractions often lead to the loss of rare alleles and allelic diversity. However, the allelic loss in homozygotes occurs faster than the loss in allelic diversity especially for alleles of low frequency (e.g. 0.01) leading to heterozygote excess (Luikart *et al*., 1998).

Each population (except Mombasa) was analysed for heterozygote deficiency or excess using BOTTLENECK version 1.2 (Cornuet and Luikart, 1998; Piry *et al*., 1999) based on 10,000 replications. Few microsatellites fit strictly into the stepwise mutation model, yet assumptions of an Infinite Allele Model may lead to occurrence of a bottleneck without the attendant heterozygosity excess (Piry *et al*., 1998; Garza and Williamson, 2001). Also, one third of the microsatellites developed for this work were compound markers involving two different motifs, enabling allele differences that are not products of the motif length (Table 2.1) (Bull *et al*., 1999). The Two Phase Model (TPM) was therefore used assuming 50 % Infinite Allele mutation model (IAM) model was therefore used, with 10,000 replications. Demographic decisions were based on Wilcoxon's Test for the probability of heterozygosity excess and deficiency and two tailed tests of both events.

Population structure analysis

Pair-wise genetic distances based on Nei's (1973) minimum genetic distance between pairs of populations was done based on all 21 alleles and restricted to alleles that were in HWE in the reference Teupasenti population alone. Population clustering was analysed based on 21 loci meeting the HWE threshold. We used Bayesian methods implemented in the program STRUCTURE version 2.2 (Pritchard *et al*., 2000; Falush *et al*., 2003, 2007) to test for the existence of a genetic structure between and within the 13 geographic populations of *T. nigrescens* genotyped. This method uses a parametric genetic mixture modelling to infer the number of populations and likelihood of assignment of individuals to each population based on allele frequency. It assumes that there are *K* populations contributing to the gene pool of the genotyped population. Each population has an unknown but characteristic allele frequency at each locus. Assuming that the loci are in Hardy Weinberg Equilibrium and are unlinked, the individuals are assigned probabilistically to one of the assumed populations depending on their genotype, such that the equilibrium is not violated. The number of populations modelled to change between known bounds and the proportional probability of membership of each individual is determined. The number of clusters of populations (the most probable *K*) is then determined based on the assignment of individuals and estimates of the allele frequencies.

Three modelling approaches were used to infer the log likelihoods of the number of clusters, two *ad hoc* (Evanno *et al*., 2005; Pritchard *et al*., 2007) and one learning approach (Beaumont *et al*., 2001). It was assumed that each group composed of unknown subgroups and that putative population identity of the insects were genetically uninformative. Any individual was equally likely to belong to any subgroup, but the number of subgroups was unknown and gene frequencies were correlated. The structure was run in four replicates per assumed *K* (between 1 - 20). An admixture model was assumed, with default parameters advised in the program manual (migration prior of 0.05; initial value for alpha inference = 1) (Pritchard *et al*., 2007). A different *lambda* was inferred for each population from the genotype data. Burn-in period was fixed at 10,000 and running length to 100,000 under admixture ancestry model. This simulation was run on the original data (all polymorphic loci, some populations not in HWE) with null alleles coded as recessive alleles (Falush *et al*., 2007).

Most individuals in this run were strongly assigned to one population (for *K* = 2 to *K* = 9), showing the existence of some population structure hence the need to estimate the true *K* (Pritchard *et al*., 2007). The probability of *K* [Ln P(D)] was plotted against values of assumed *K* between 1- 20, predicting the true value of *K* (Figure 7.1) as the point where the curve enters the plateaus phase (Pritchard *et al*., 2007).

Finally, the approach of Evanno (2005) was used on the results of the first method to determine the most likely *K*, and to compare with the result obtained according to the user's STRUCTURE manual (Pritchard *et al*., 2007). Briefly, we analysed second order rate of change of *L*(*K*), denoted as 'Ln P(D)' in the simulation results, depicting the true *K* as the point where there was the sharpest change in the slope of the distribution of $L(K)$. This was determined by plotting ΔK= against assumed K values from the simulation (Figure 7.2):

$\Delta K = m(|L''K|)/s[L(K)]$

Where ΔK is an *ad hoc* quantity based on the second order rate of change of the likelihood function with respect to K. It is the mean absolute value of $L''K$ between the runs divided by the standard deviation of $L(K)$ for each value of K $\{s[L(K)]\}$.thus:

$L''K = L'(K+1)\text{-}L'(K)$

The mean differences between the successive values of likelihood of K $[L'(K)]$ are computed as:

$L'K = L(K) - L(K\text{-}1)$

In the third approach to determine K, the reference population method (PopFlag = 1 option) of Beaumont *et al*. (2001) was used. Since in the first run, the Yoro geographical population clustered neatly together across all putative vales of K, they were used as a learning sample assumed to be correctly assigned to their genetic population. Modelling of other populations was therefore based on the *a priori* assumption that these samples were correctly classified. This approach improves the accuracy of classification and inference of genetic clusters, but would be less reliable if clusters are not very distinct genetically (Pritchard *et al*., 2007).

7.4 Results

7.4.1 Genetic diversity

For the gene diversity tests, only the 16 loci that were polymorphic and in HWE in the large populations are shown (Table 7.1). Most microsatellite loci were polymorphic, with a mean of 3.8 alleles per locus. All samples considered together showed significant deviation from Hardy Weinberg equilibrium at all loci. Specific population parameters were variable (Table 7.2). Although most geographic populations did not deviate significantly from the HWE expectations for most alleles, a few populations either deviated significantly or were monomorphic for alleles that were polymorphic and in equilibrium for other populations. This was most common in small populations from which of less than 10 individuals were sampled. The unbiased estimates of genetic differentiation are summarised on Table 7.2. Most samples from Mombasa failed to amplify at most loci after three attempts and hence the population was excluded from bottleneck and genetic diversity analyses as the general failure may have been caused by poor DNA quality.

The analysis using MICROCHECKER detected significant existence of null alleles in five loci for the test population. The mean frequency of null alleles was 0.25 ± 0.14 including individuals that did not amplify across most alleles, while the mean number of completely genotyped individuals per population and locus was 13.5 ± 10.1. The Benin population had the highest proportion of completely genotyped individuals, while the small populations (Kiboko and Yoro) did not have a single sample completely genotyped at all loci.

7.4.2 <u>Genetic differentiation</u>

Pair-wise genetic distances between pairs of geographic populations were higher between the southern (Honduras/Costa Rica populations) and Mexico populations than within these general groupings (Table 7.3). However, these distances varied significantly within these population groups, especially among populations obtained from laboratory colonies or with recent such ancestry.

7.4.3 <u>Cluster analysis</u>

Without *a priori* assumptions on population origin and by coding null alleles as recessive, the analysis of the potential substructure in the pooled population based on 432 individuals and all 21 polymorphic loci resulted in a maximum of nine genetic clusters, with individuals tending to be assigned to one grouping. The curve of the posterior probabilities of K against assumed K, used as an informal *ad hoc* indicator of the number of genetic populations (Pritchard *et al*., 2007) rose till K = 5 and then levelled off (Figure 7.1). Thus the values of real K could be visually discerned at approximately K = 5. Using the second order change in the rate of the rise in the posterior probabilities, the most likely number of clusters was estimated at 5 (Figure 7.2). The *a priori* set reference population flag approach of Beaumont *et al*. (2001) did not reveal a significant structure as all individuals were allocated to the possible clusters in equal proportions between K = 2 to K = 9.

The results of the cluster analysis were in agreement with the proportion of membership of populations to each of the clusters (Figure 7. 3). The five inferred clusters generally reflected the geographical origin of the sampled populations. Cluster 1 included Batan, Tlaltizaspan and Mombasa samples (Mexican origin), cluster 2 Benin samples and cluster 3 Kiboko, KARI and “Store” populations. Cluster 4 grouped together Malawi and Ghana samples, while

Cluster 5 had all the populations from Honduras. Oaxaca geographic population was allocated partially to clusters 1 to 3, and 1, 3 and 4, respectively. The lowest proportion of membership of a given cluster was 0.00 for Kiboko to clusters 1 and 2 and Gualaso to clusters 1 and 3 and the highest was 0.996 in cluster 3 for the Kiboko population. The highest assignments were generally from small populations and colonies with a distinct laboratory culture history.

7.4.4 Demographic history

Analysis of possible genetic signatures of population bottlenecks and expansion revealed a significant mode-shift in the Gualaso population only. This is however not informative, since this was a very small population (n = 5). The rest of the populations showed allele frequency distributions that were typically hyperbolic, indicating no loss of rare alleles. Most populations showed significant heterozygote deficiency at more loci than those that were excess, leaving a signature of recent population expansion, but without a significant mode shift.

Table 7.1: Summary of genetic variation at 16 neutral microsatellite loci in twelve *Teretrius nigrescens* geographic populations originally from Mexico, Honduras Costa Rica.

	Mexico			Ex Mexico			Ex Costa Rica			Honduras		
	Batan	Tlaltizaspan	Oaxaca	KARI	Kiboko	Store	Benin	Ghana	Malawi	Teupasenti	Gualaso	Yoro
TnM36												
N	37	50	35	31	17	8	60	36	34	85	5	8
n_A	6	6	2	2	4	1	4	4	5	3	2	3
n_R	2.877	2.891	1.868	2.311	1.807	2.524	1.000	2.688	3.243	2.686	2.000	2.617
H_E	0.583	0.589	0.284	0.225	0.415	0.000	0.521	0.511	0.496	0.539	0.320	0.461
H_O	0.514	0.560	0.171	0.194	0.294	0.000	0.617	0.528	0.529	0.541	0.400	0.500
F_{IS}	0.133	0.059	0.409	0.155	0.319	------	-0.176	-0.019	-0.053	0.001	-0.143	-0.018
TnM51												
N	38	50	35	29	17	8	60	36	36	85	5	8
n_A	3	4	3	2	1	1	3	1	2	2	1	2
n_R	1.263	1.752	1.642	1.548	1.427	1.000	1.000	1.000	1.139	1.705	1.000	2.000
H_E	0.052	0.151	0.135	0.098	0.000	0.000	0.125	0.000	0.027	0.199	0.000	0.469
H_O	0.053	0.120	0.143	0.035	0.000	0.000	0.133	0.000	0.028	0.153	0.000	0.250
F_{IS}	-0.007	0.217	-0.043	0.659	------	------	-0.055	------	0.000	0.235	------	0.517
TnM54												
N	28	39	26	30	17	8	56	35	35	85	5	8
n_A	5	7	1	3	2	2	5	2	5	2	1	1
n_R	3.507	3.737	1.000	1.439	2.269	1.999	1.964	1.267	1.642	1.059	1.000	1.000
H_E	0.584	0.625	0.000	0.331	0.493	0.305	0.087	0.056	0.135	0.012	0.000	0.000
H_O	0.071	0.180	0.000	0.400	0.294	0.375	0.071	0.057	0.086	0.012	0.000	0.000
F_{IS}	0.882	0.719	------	-0.192	0.429	-0.167	0.185	-0.015	0.378	0.000	------	------
TnM55												
N	38	50	35	31	17	8	56	36	36	75	5	8
nA	11	9	6	4	2	3	8	2	2	6	2	3
nR	3.586	4.091	3.748	2.464	2.805	1.847	2.625	1.666	1.260	2.865	2.000	2.500
H_E	0.621	0.649	0.638	0.544	0.251	0.539	0.459	0.176	0.054	0.593	0.500	0.320
H_O	0.500	0.440	0.657	0.419	0.294	0.750	0.179	0.139	0.000	0.333	0.200	0.375
F_{IS}	0.207	0.331	-0.016	0.244	-0.143	-0.333	0.617	0.222	1.000	0.443	0.667	-0.105

	Mexico			Ex Mexico			Ex Costa Rica			Honduras		
	Batan	Tlaltizaspan	Oaxaca	KARI	Kiboko	Store	Benin	Ghana	Malawi	Teupasenti	Gualaso	Yoro
TnM70												
N	38	49	35	30	17	8	59	36	36	79	5	8
n_A	8	8	5	6	2	1	4	2	3	3	2	2
n_R	5.173	4.730	3.039	2.293	3.132	1.959	1.000	1.762	2.362	2.866	2.000	1.964
H_E	0.817	0.770	0.479	0.603	0.360	0.000	0.341	0.219	0.527	0.614	0.420	0.305
H_O	0.632	0.714	0.429	0.567	0.353	0.000	0.322	0.139	0.472	0.342	0.600	0.375
F_{IS}	0.239	0.083	0.119	0.077	0.050	------	0.064	0.377	0.119	0.448	-0.333	-0.167
TnM71												
N	37	49	34	30	16	8	58	36	36	85	5	8
n_A	6	6	6	2	2	2	4	3	2	3	2	2
n_R	3.344	3.835	3.861	2.688	2.317	1.797	1.625	1.926	1.762	2.948	2.000	1.999
H_E	0.562	0.667	0.699	0.473	0.219	0.117	0.500	0.224	0.219	0.663	0.320	0.430
H_O	0.432	0.612	0.471	0.500	0.250	0.125	0.466	0.194	0.139	0.459	0.400	0.625
F_{IS}	0.244	0.092	0.340	-0.041	-0.111	0.000	0.078	0.146	0.377	0.313	-0.143	-0.400
TnM72												
N	38	50	35	30	17	8	60	36	36	81	5	8
n_A	8	7	9	7	2	2	8	5	3	7	3	4
n_R	4.788	4.233	5.119	3.561	4.044	1.847	1.625	2.117	2.552	3.000	3.000	3.214
H_E	0.750	0.739	0.797	0.734	0.251	0.117	0.579	0.230	0.474	0.528	0.540	0.484
H_O	0.658	0.740	0.743	0.533	0.294	0.125	0.600	0.139	0.417	0.469	0.600	0.375
F_{IS}	0.136	0.009	0.082	0.289	-0.143	0.000	-0.029	0.407	0.135	0.118	0.000	0.288
TnM79												
N	36	46	31	27	9	8	56	32	34	78	5	8
n_A	6	5	3	3	2	1	3	3	4	4	2	2
n_R	2.757	2.721	2.222	2.157	2.326	1.977	1.000	2.088	2.770	2.926	2.000	1.875
H_E	0.397	0.386	0.380	0.524	0.346	0.000	0.305	0.366	0.582	0.636	0.180	0.219
H_O	0.167	0.130	0.065	0.000	0.000	0.000	0.089	0.031	0.206	0.231	0.200	0.000
F_{IS}	0.589	0.668	0.835	1.000	1.000	------	0.712	0.917	0.655	0.641	0.000	1.000
TnM85												
N	38	50	35	28	16	8	59	36	35	84	5	8
n_A	12	12	9	6	4	4	8	4	4	8	2	3
n_R	5.150	5.276	3.579	3.754	3.860	2.758	3.214	2.673	2.928	3.420	2.000	2.617
H_E	0.776	0.820	0.511	0.615	0.373	0.484	0.689	0.489	0.580	0.545	0.480	0.461
H_O	0.790	0.720	0.371	0.679	0.188	0.625	0.746	0.556	0.486	0.452	0.400	0.250
F_{IS}	-0.004	0.132	0.287	-0.085	0.521	-0.228	-0.073	-0.122	0.177	0.176	0.273	0.509

	Mexico			Ex Mexico			Ex Costa Rica			Honduras		
	Batan	Tlaltizaspan	Oaxaca	KARI	Kiboko	Store	Benin	Ghana	Malawi	Teupasenti	Gualaso	Yoro
TnN14												
N	35	46	32	30	13	8	60	32	34	81	5	8
n_A	6	8	6	3	4	2	3	1	2	3	1	1
n_R	4.556	4.302	3.748	1.392	2.760	3.259	1.875	1.000	1.147	1.182	1.000	1.000
H_E	0.759	0.695	0.578	0.549	0.642	0.219	0.081	0.000	0.029	0.037	0.000	0.000
H_O	0.743	0.739	0.563	0.533	0.769	0.250	0.083	0.000	0.029	0.012	0.000	0.000
F_{IS}	0.036	-0.053	0.042	0.046	-0.159	-0.077	-0.024	------	0.000	0.665	------	------
TnN21												
N	38	50	35	31	17	8	60	36	36	79	5	8
n_A	6	5	4	2	2	2	3	4	2	5	1	3
n_R	2.111	2.262	1.553	2.040	1.405	1.294	1.625	2.258	1.999	1.664	1.000	2.589
H_E	0.239	0.428	0.110	0.062	0.057	0.117	0.395	0.345	0.500	0.143	0.000	0.398
H_O	0.184	0.400	0.057	0.000	0.059	0.125	0.383	0.278	0.583	0.089	0.000	0.250
F_{IS}	0.242	0.075	0.491	1.000	0.000	0.000	0.039	0.207	-0.154	0.387	------	0.429
TnN28												
N	38	50	35	31	17	8	58	36	36	82	5	8
n_A	5	9	6	6	4	3	4	4	5	3	2	1
n_R	1.949	2.271	2.047	1.941	2.788	2.170	2.589	2.117	1.886	1.875	2.000	1.000
H_E	0.196	0.241	0.233	0.456	0.261	0.398	0.219	0.269	0.182	0.244	0.180	0.000
H_O	0.211	0.240	0.086	0.194	0.294	0.500	0.035	0.194	0.139	0.232	0.200	0.000
F_{IS}	-0.063	0.013	0.64	0.587	-0.096	-0.191	0.845	0.29	0.249	0.058	0.000	------
TnN2												
N	37	49	34	28	10	8	60	34	36	79	5	8
n_A	3	8	4	3	3	3	3	4	2	3	2	1
n_R	2.102	3.172	2.289	2.730	3.114	2.500	2.589	2.705	1.919	1.127	2.000	1.000
H_E	0.414	0.606	0.507	0.653	0.535	0.398	0.516	0.412	0.330	0.025	0.320	0.000
H_O	0.460	0.612	0.471	0.536	0.600	0.500	0.617	0.412	0.417	0.025	0.000	0.000
F_{IS}	-0.097	-0.001	0.087	0.197	-0.069	-0.191	-0.187	0.016	-0.250	-0.003	1.000	------
TnN1												
N	34	49	32	29	13	8	59	33	36	79	5	8
n_A	3	9	4	3	3	3	4	5	2	3	2	1
n_R	2.425	3.366	2.443	2.876	2.940	2.376	2.589	2.960	1.964	1.302	3.000	1.000
H_E	0.443	0.613	0.535	0.647	0.476	0.398	0.527	0.509	0.389	0.062	0.340	0.000
H_O	0.412	0.571	0.344	0.586	0.462	0.500	0.542	0.424	0.472	0.013	0.200	0.000
F_{IS}	0.085	0.078	0.371	0.111	0.071	-0.191	-0.020	0.181	-0.202	0.797	0.500	------

	Mexico			Ex Mexico			Ex Costa Rica			Honduras		
	Batan	Tlaltizaspan	Oaxaca	KARI	Kiboko	Store	Benin	Ghana	Malawi	Teupasenti	Gualaso	Yoro
TnM40												
N	37	50	35	31	17	8	60	36	35	80	5	8
n_A	3	9	4	3	3	3	4	5	2	4	2	1
n_R	4.330	4.574	4.293	3.082	3.081	3.868	1.625	2.917	2.805	2.907	2.000	2.875
H_E	0.744	0.746	0.751	0.586	0.609	0.117	0.617	0.639	0.611	0.620	0.320	0.602
H_O	0.378	0.580	0.457	0.452	0.706	0.125	0.467	0.389	0.343	0.625	0.000	0.625
F_{IS}	0.501	0.232	0.404	0.245	-0.129	0.000	0.252	0.403	0.450	-0.002	1.000	0.028
TnM41												
N	38	50	34	31	15	8	60	36	35	83	5	8
n_A	6	6	6	4	2	2	6	4	5	5	2	2
n_R	4.684	4.734	3.470	3.750	2.425	1.999	1.875	2.493	2.218	2.341	2.000	1.999
H_E	0.781	0.796	0.661	0.456	0.480	0.219	0.692	0.514	0.409	0.503	0.500	0.430
H_O	0.684	0.760	0.677	0.548	0.533	0.250	0.700	0.528	0.486	0.542	0.600	0.625
F_{IS}	0.136	0.055	-0.008	-0.186	-0.077	-0.077	-0.003	-0.014	-0.172	-0.072	-0.091	-0.400
FIS All	0.223	0.165	0.233	0.194	0.103	-0.175	0.099	0.205	0.143	0.246	0.248	0.138

N: number of individuals genotyped per locus

n_A: number of alleles per locus, n_R Allelic richness per locus,

H_E: expected heterozygosity, H_O: Observed heterozygosity,

F_{IS}: Weir and Cockerham's (1984) estimate of Wrights (1951) fixation index.

Table 7.2: Estimates of Nei's unbiased gene diversity of 13 *Teretrius nigrescens* populations (columns) at 21 polymorphic loci (rows).

	Batan	Benin	Kiboko	Ghana	Gualaso	KARI	Malawi	Mombasa[1]	Oaxaca	Store	Teupasenti	Tlaltizaspan	Yoro
TnM36	0.595	0.524	0.432	0.518	0.350	0.246	0.503	NA	0.290	0.000	0.538	0.597	0.491
TnM47	0.648	0.339	0.605	0.028	0.675	0.314	0.033	1.000	0.513	0.643	0.386	0.700	0.000
TnM48	0.655	0.168	0.071	0.442	0.600	0.092	0.509	0.500	0.225	0.125	0.780	0.676	0.759
TnM51	0.050	0.123	0.000	0.000	0.000	0.098	0.028	0.667	0.137	0.000	0.214	0.156	0.518
TnM53	0.661	0.683	0.710	0.592	0.900	0.548	0.677	NA	0.242	0.482	0.682	0.639	0.750
TnM54	0.589	0.085	0.515	0.056	0.000	0.352	0.138	0.000	0.000	0.321	0.022	0.640	0.000
TnM55	0.689	0.485	0.257	0.223	0.600	0.574	0.056	0.750	0.648	0.563	0.605	0.648	0.339
TnM70	0.847	0.361	0.371	0.213	0.450	0.626	0.537	NA	0.487	0.000	0.643	0.785	0.321
TnM71	0.562	0.526	0.225	0.263	0.350	0.514	0.223	NA	0.713	0.125	0.731	0.664	0.446
TnM72	0.801	0.587	0.257	0.322	0.600	0.747	0.482	0.667	0.809	0.125	0.576	0.745	0.527
TnM79	0.406	0.320	0.389	0.377	0.200	0.548	0.596	NA	0.392	0.000	0.645	0.400	0.250
TnM85	0.791	0.695	0.392	0.495	0.550	0.651	0.590	1.000	0.521	0.509	0.572	0.822	0.509
TnN14	0.771	0.080	0.663	0.000	0.000	0.581	0.029	NA	0.587	0.232	0.036	0.710	0.000
TnN21	0.327	0.426	0.059	0.350	0.000	0.092	0.506	NA	0.112	0.125	0.182	0.429	0.438
TnN28	0.270	0.212	0.268	0.281	0.200	0.457	0.185	NA	0.238	0.420	0.270	0.248	0.000
TnN2	0.443	0.519	0.561	0.418	0.400	0.675	0.333	0.500	0.516	0.420	0.048	0.607	0.000
TnM23	0.898	0.782	0.753	0.583	0.200	0.774	0.737	0.833	0.873	0.500	0.616	0.795	0.125
TnN1	0.478	0.553	0.497	0.518	0.400	0.658	0.393	NA	0.546	0.420	0.060	0.621	0.000
TnM40	0.779	0.639	0.625	0.670	0.400	0.597	0.624	0.917	0.767	0.125	0.646	0.764	0.643
TnM41	0.796	0.694	0.495	0.522	0.550	0.486	0.414	NA	0.671	0.232	0.504	0.804	0.446
TnM66	0.564	0.541	NA	0.558	0.500	0.703	0.622	NA	0.123	0.667	0.352	0.491	0.286

[1]Inadequate amplification and sample size

Table 7.3: Multilocus Nei's minimum distance between pairs of populations based on all 21 polymorphic loci (above diagonal) and on 16 neutral markers (in HWE in Teupasenti reference population) including null alleles (below diagonal)

	Batan	Benin	Kiboko	Ghana	Gualaso	KARI	Malawi	Oaxaca	Store	Teupasenti	Tlaltizaspan	Yoro
Batan		0.143	0.177	0.226	0.173	0.105	0.252	0.065	0.276	0.244	0.005	0.235
Benin	0.084		0.270	0.061	0.097	0.197	0.095	0.080	0.329	0.118	0.097	0.109
Kiboko	0.121	0.161		0.351	0.416	0.181	0.370	0.212	0.384	0.380	0.177	0.441
Ghana	0.135	0.030	0.190		0.171	0.316	0.058	0.207	0.465	0.147	0.177	0.163
Gualaso	0.147	0.069	0.282	0.087		0.227	0.173	0.150	0.446	0.042	0.135	0.069
KARI	0.076	0.121	0.117	0.198	0.177		0.321	0.096	0.162	0.274	0.108	0.296
Malawi	0.160	0.046	0.220	0.026	0.096	0.211		0.243	0.457	0.150	0.195	0.180
Oaxaca	0.045	0.044	0.131	0.108	0.111	0.064	0.138		0.234	0.187	0.055	0.200
Store	0.213	0.211	0.217	0.275	0.229	0.104	0.292	0.149		0.348	0.248	0.509
Teupasenti	0.143	0.056	0.238	0.066	0.041	0.165	0.069	0.097	0.209		0.201	0.061
Tlaltizaspan	0.012	0.059	0.132	0.110	0.126	0.083	0.126	0.040	0.206	0.118		0.187
Yoro	0.180	0.066	0.290	0.075	0.053	0.221	0.091	0.130	0.294	0.040	0.152	

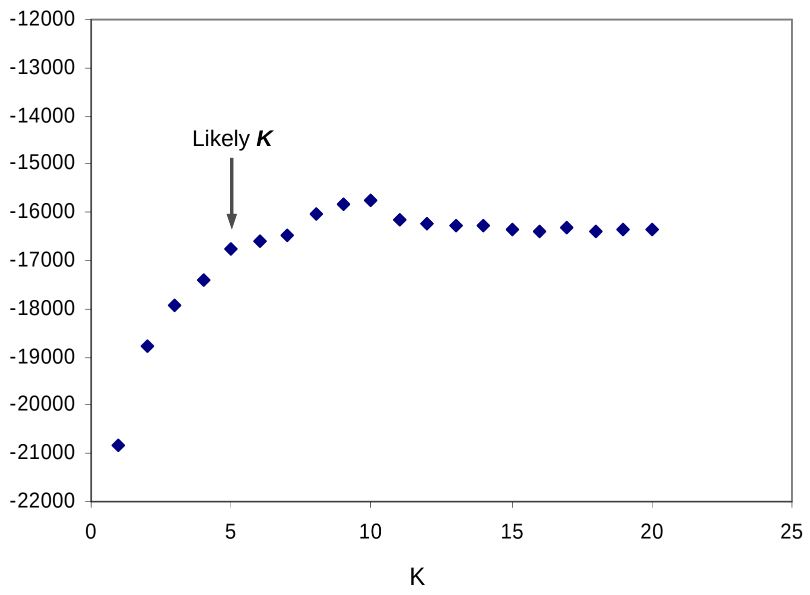


Figure 7.1: Estimation of the population structure of *T. nigrescens* based on *ad hoc* measure of the gradient of *K* against L(*K*) (Pritchard *et al*., 2007).

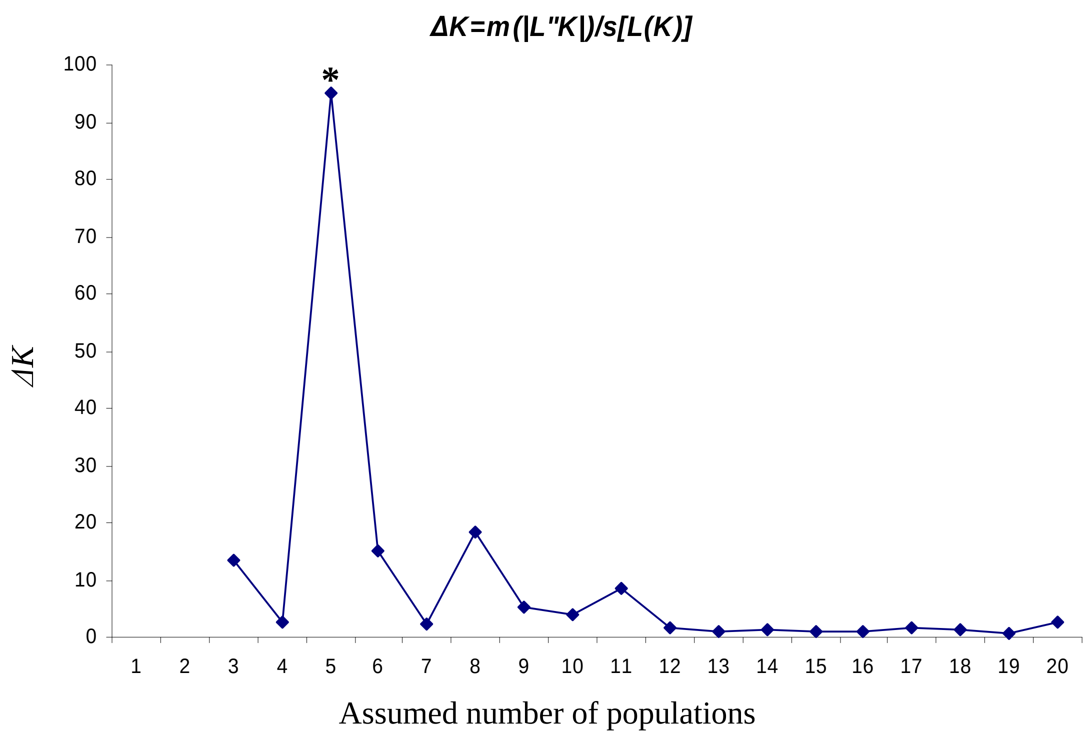
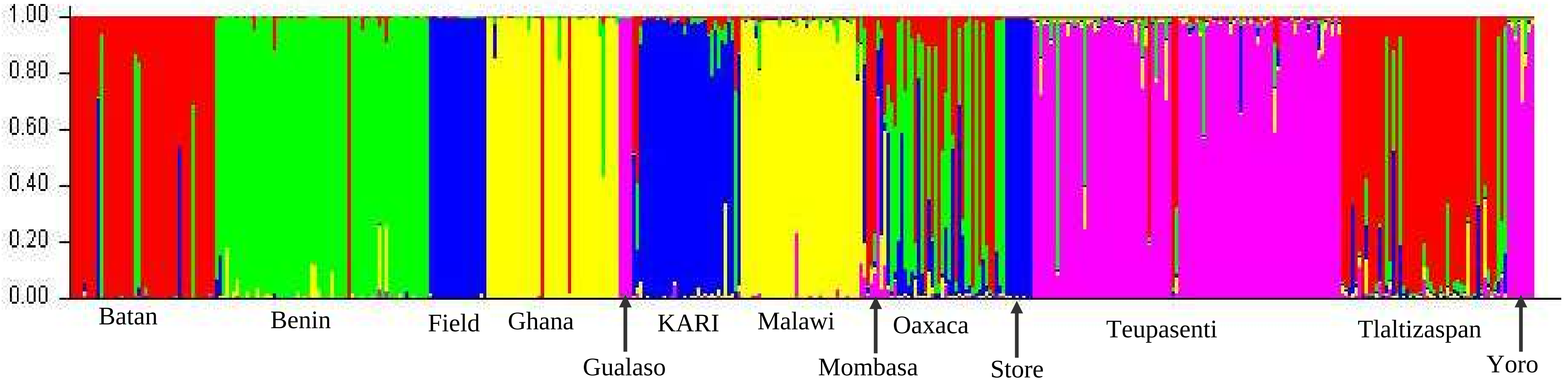


Figure 7.2: Detection of the number of clusters of individuals based on the *ad hoc* measure (ΔK) (Evanno *et al*., 2005). The asterisk marks the inferred *K*. sharp changes in gradient in after K = 5 suggests the existence of genetic substructure.



Cluster	Batan	Benin	Kiboko	Ghana	Gualaso	KARI	Malawi	Mombasa	Oaxaca	Store	Teupasenti	Tlaltizaspan	Yoro
1	0.889	0.021	0.000	0.054	0.000	0.058	0.007	0.430	0.362	0.003	0.033	0.801	0.004
2	0.079	0.952	0.000	0.022	0.001	0.054	0.022	0.085	0.491	0.004	0.039	0.116	0.034
3	0.030	0.004	0.996	0.004	0.000	0.863	0.005	0.218	0.125	0.985	0.010	0.043	0.004
4	0.001	0.021	0.002	0.921	0.200	0.018	0.949	0.138	0.014	0.004	0.022	0.024	0.057
5	0.001	0.002	0.001	0.000	0.798	0.007	0.016	0.130	0.009	0.004	0.896	0.016	0.901

Figure 7.3: Results of cluster analysis using by Bayesian methods on STRUCTURE software. Each vertical bar represents the coefficient of a genotyped individual's membership to one of the five inferred genetic clusters: red- Mexico group (Batan, Tlaltizaspan); Green- Costa Rica Group (Benin); Yellow- Malawi/Ghana subpopulations; Blue- KARI Group (KARI, Store, Kiboko); and Pink - Honduras group (Teupasenti, Gualaso, Yoro).
The table below the distruct plot shows the mean proportion of individual membership to each cluster.

7.5 Discussion

Most samples in this study were unambiguously genotyped at most loci. Null alleles were relatively rare, as seen in the HWE expectations, and, were not corrected for in the measures of genetic distance. Other studies have shown little difference between genetic analyses correcting for null alleles and those that do not (Liu *et al*., 2009; Williams *et al*., 2005). A deviation from HWE expectations was observed in a number of allele-population combinations. All analyses combining more than one putative population as defined by geographical or sampling source resulted in a decreased adherence to HWE expectations. Failure to clearly genotype some samples or loci may be attributed to null alleles (i.e., a mutation within the primer annealing region) or failure of PCR due to the quality of DNA. Amplification failure due to null alleles are usually occurs at few loci only. Poor template DNA quality causes poor amplification across most loci in specific samples (Dakin and Avise, 2004). Null alleles may cause a shift from HWE, often leading to heterozygosity deficiency as many heterozygotes are coded as homozygotes (Liu *et al*., 2009).

The genetic diversity, uncorrected for population size, did not show any trends between laboratory and field populations. The Mexican populations however had greater diversity within populations than the Honduras and Costa Rica populations. Genetic diversity was however higher in parent laboratory than in possible daughter sample populations (e.g. Benin>Malawi or Ghana; KARI > Kiboko, Store, Mombasa). Such trends indicate genetic drift, sampling effects or loss of some alleles during colony subdivision when establishing new colonies (Lloyd *et al*., 2005).

A further analysis of possible bottlenecks in the population suggests that most populations were expanding, but without a genetic bottleneck signature. Such population disturbances are common in biological invasions, range expansion and insect rearing (Bigler, 1992; Memmott *et al*., 2005). The ability to detect a population bottleneck signature based on the heterozygosity excess and deficiency alone has been questioned (Garza and Williamson, 2001; Cournet *et al*., 1998). This analysis is dependent on prior assumptions of microsatellite mutation models, which are often not studied directly. While there is no general consensus for the correct mutation model to use and most scientists adopt the middle ground assumption of the TPM model, the different models gave contrasting results. Within two phase model (TPM), one must make a quasi arbitrary decision about the proportion of loci in the single step and infinite allele mutation models. The choice of one or the other mutation model determines the result and decision obtained. The correct model is only correctly discernible through mating studies and sequencing of alleles (Ellegren, 2004). In this study, one third of the alleles used were composite, some composed of different repeat core sizes. Though several studies have identified such markers, they are clearly not accounted for in the three mutation models available. The inability of the He/Ho ratio to detect bottlenecks has been reported in populations with known recent bottleneck events and within the 'window for testing' for such events (Lawler, 2008). In this study, the Kiboko population, raised from just three adults, showed signs of expansion but not a bottleneck within one year of culturing, possibly resulting from the masking of the signal during subsequent population expansion.

In biological control agents, the most probable population constrictions are likely to occur during isolation from the field, laboratory rearing, subdivision and lag phase after release. Under artificial rearing, these events are often followed by leading to a population expansion during rearing. Generally, population bottlenecks result in either population extirpation or if there is recovery, an expansion. Consequently heterozygosity deficiency is observed especially in a rapidly growing population (Bonhomme *et al*., 2008). In fact, in this study, the program could not detect any bottleneck signal well within the bottleneck detection window (Lawler, 2008). Sub culturing is known to have been done for further releases (Meikle *et al.*, 2002). Insects released in Ghana, Malawi, Tanzania and Nigeria originated from the Benin population (Anonymous, 1999). Similarly, Benin and Ghana samples had a recent history of introgression with wild individuals caught in Africa, but virtually none from Central America, the predator's native range (Meikle *et al.*, 2002). This lack of secondary isolation and reintroductions from Mexico was possibly partly to maintain colony purity and avoid contamination of laboratory colonies with potentially diseased field samples. The observation of greater heterozygosity deficiency in most laboratory populations compared to the Teupasenti sample – a recent field isolation – thus indicates a recent population expansion that could mask the effect of population bottlenecks. Further, *T. nigrescens* are reared in jars, often with a starting population of 20 adults of around 10 females. Rearing insects in sealed rearing jars creates an effective population fragmentation that might lead to allelic drifts, especially if colonies from different jars are not mixed after every few generations between jars. This would thus create a series of slight bottlenecks fragmentation during rearing and a significant founder effect at every sub-culturing. Consequently, loss of rare alleles and increased homozygosity may lead to the over-expression of deleterious genes, depletion of rare but important fitness linked alleles and increased sensitivity to environmental fluctuations or pathogens, reducing the potential establishment and success of eventual released individuals. Indeed, our data shows a general reduction in numbers and allelic richness among laboratory populations compared to the field populations.

The genetic mixture analysis revealed a clear genetic structure of *T. nigrescens* following the geographical distribution. Two methods of estimating the number of populations gave consistent results, with an indication of existence of a hierarchical structure. Five distinct populations were estimated with the Oaxaca population showing a mixed ancestry at this level. Although the estimation of *K* in such a case is informative, it is more important to figure out what level of *K* is most biologically informative (Evanno *et al*., 2005; Pritchard *et al*., 2007). For example, although structure revealed hidden genetic substructure and demographic history events in cat and insect populations (Beaumont *et al*.; 2001, Liu *et al*., 2008), it could not account for certain known demographic events among Dexter cattle breeds (Bray *et al*., 2009). In this study, the assumption of two clusters (*K* = 2) grouped together all populations from Mexico with those of Mexican origin (KARI, Kiboko and “Store” and Mombasa), while those originating from all other locations formed the other cluster. At *K* = 3, the southern cluster was subdivided into two sub clusters: Honduras (Gualaso, Teupasenti and Yoro) and Costa Rica (Benin, Ghana and Malawi). This reflected the correct countries of initial origin of these populations. At *K* = 4, Mexican populations were further divided into two clusters: the first cluster contained samples from Batan, Tlaltizaspan and Oaxaca (recently sampled from the field), while the second included samples of Mexican origin released in Kenya in the 1990s. The distinction between the Costa Rican populations released in Ghana, Zambia and Malawi from the mother population maintained in Benin with little evidence frequent field introgressions was evident at *K* = 5. Beyond *K* = 5, subdivisions occurred but the Honduras populations always ended up in the same cluster.

This hierarchical mode of clustering is informative. First, one is able to detect the origin of samples used, corroborating the reconstruction of the history of biological control in Africa. Also, there was evidence of allelic diversity shifts in culture after separation for just a few years (Hufbauer *et al*., 2004; Lloyd *et al*., 2005). Changes in allelic diversity and composition have been observed in laboratory colonies of *Cotesia flavipes* (Omwega and

Overholt, 1996) and *Aphidius ervi* (Hufbauer *et al*., 2002; 2004). However, the association between these neutral changes and fitness-related traits is debatable (van Tienderen *et al*., 2002; Bekessy *et al*., 2003). Due to logistical stringencies and because of their sheer rarity in the field, natural enemies are often collected as small subpopulations ill-representing the full genetic diversity in the source population (Lloyd *et al*., 2005). Most of the populations released in Africa have been separated from the mother populations for about 20 years now (Giles *et al*., 1996; Meikle *et al*., 2003). This period corresponds to less than 300 generations of *T. nigrescens*, which is comparatively short in species evolution. The effect of genetic drift and founder effects may be more in play considering the rearing protocol of fragmented populations. Other than a small study population from mid-altitude Mexico introduced into the laboratories of IITA in 1990s (W. M. Meikle, USDA, Weslaco, USA, personal communication), there is little evidence of introgression of the gene pool using samples collected over time or from different geographical locations. This could be the main contributing factor to the hierarchical structure and maintenance of genetic distinctiveness of the populations recovered.

The practical role of release sizes in the success of biological control establishments is controversial (Hopper and Roush, 1993; Grevstad, 1999; Memmott *et al*., 2005). The genetic diversity of natural enemies is a key parameter in biological control and a possible determinant of their eventual establishment, ecological fitness and effectiveness. Rapid adaptation to local environmental conditions and eventual effectiveness of may not only depend on the suitability of the species introduced, but also the existence of well adapted geographical biotypes (Diehl and Bush, 1984). In fact, it has been recognised that variability of natural enemies is invaluable in biological control in providing pools of genes on which selection occurs (Roush, 1990; Roderick and Navajas, 2003). The process of collection, quarantine rearing, importation and release may impose series of bottlenecks leading to loss of genetic variation (Hopper and Roush, 1993; Hopper *et al*., 1993; Lloyd *et al*., 2005; Fauvergue *et al*., 2007). Often, one starts with a small population losing on potentially important rare alleles (Nei *et al*., 1975; Bigler, 1992). Rearing protocols aiming at producing pure populations, long time laboratory culturing and subdivision may reduce the internal diversity of the natural enemies. Where information was available, new colonies and releases were shown to be founded by between 200 and 5000 individuals respectively (Anonymous, 1999; P. Likhayo, KARI; H.Z. Irmgard, IITA, personal communication). On the face value, these numbers meet the minimum viable population size criterion for diploid organisms, but such diversity must take into account underlying genetic variability (Rai, 2003). However, our data show induced systematic bottleneck/expansion or drift effects perhaps during initial isolation from the field or by rearing in fragmented non-contiguous subpopulations.

7.6 References

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CHAPTER EIGHT

General Discussion and Conclusions

The sustainable management of the invasive LGB in Africa depends on finding a suite of control measures and formulation of decision–making tools applicable in diverse environments. These should result in significant reduction in pest populations, yet be compatible with other post-harvest pest management measures. The correlation between weather conditions and the survival, abundance and flight activity of the larger grain borer has been studied before (Fadamiro and Wyatt, 1995; Hill *et al*., 2003; Hodges *et al*., 2003). Models of the flight activity generally identify warm humid conditions as being both suitable for development and initiation of flight activity of the pest (Rees *et al*., 1990; Tigar *et al*., 1994; Hodges *et al*., 2003). Other than Tigar *et al*. (1994) little modelling and prediction of the flight activity of the predator has been done. It has been documented that weather conditions may trigger flight in *P. truncatus* either directly or through their contribution to rapid population build-up leading to crowding (Fadamiro and Wyatt, 1995; Hodges *et al*., 2003). The findings of this study generally concurred with previous workers on the seasonal patterns of the LGB flight. The differences in exact model parameters under different ecological conditions suggest that local adaptation may distort the predictive ability of models previously developed. Continuous surveillance of the pest through the national agricultural research systems is necessary.

Although *T. nigrescens* established in Kenya (around the coast and semi-arid lower eastern region), its contribution to the control of the LGB in most of the areas sampled is doubtful. Giles *et al*. (1996), Nang'ayo (1996) and Hill *et al*. (2003) observed a marked reduction of the pest’s flight activity following release of the predator into a largely maize deficit area. However, Hill *et al*. (2003) recorded very low subsequent *T. nigrescens* flight activity.

Fluctuation in the LGB population may be more a result of meteorological parameters than the action of the predator. It is unlikely that a predator that is barely detectable would be an important control agent of a ubiquitous pest. Population fragmentation, post release extirpation and the dearth of safe establishment habitats could have contributed to these observations (Grevstad, 1999; Shea, and Possingham, 2000) and should be accommodated in further releases. A more numerically and genetically aggressive release strategy should also be adopted. In Kenya, about 4900 beetles in total were released per location, compared to 135,000 in Togo and 100,000 in Zambia. To increase the chances of success of the predator, a wider genetic diversity from across the predator's native range and ecological modelling and mapping studies should be incorporated.

The existence of wild populations of *T. nigrescens* in Kenya (e.g. Mombasa, Store and Field populations) is fascinating. The virtual absence of the predator in western Kenya undetectable is informative. It would be interesting to know the selective barriers to westward dispersal of the predator, since the LGB has subsequently established in these. Climatic and topographical factors may be responsible (Giles *et al*., 1996). Possibly, the introduction of the LGB into the western part of the country was through independent anthropogenic causes such as commerce. Studying the population differentiation of the LGB, using molecular markers might elucidate the origin of the infesting populations countrywide.

The role of micro evolution in the establishment of the LGB in Africa should be investigated. The levels of grain damage and environment of the pest in Africa are quite different from that observed in the native range (Rees *et al*., 1990; Farrel and Schulten, 2002). The successful establishment of a pest often depends on its ability to adapt to new ecological conditions (Hufbauer and Roderick, 2005). Rapid changes in ecology and genetic diversity are recognised as the key to success of an invasion (Lee, 2002). Rapid selection of a subtype of the invader occurs, leading to the survival of better adapted individuals following the lag phase of the invasion. Such changes probably favoured adaptation to the farm-store environment unlike the semi wild founder populations. In this study, pest recoveries were higher nearer roads, markets and human dwellings. One issue still unresolved is the main hosts of the LGB in the wild (Nang'ayo *et al*., 1993; Nansen *et al*., 2001). The role of local fauna in the perennation of the pest in Africa should be studied to incorporate sound environmental management in its control.

The hypothesis that biologically discontinuous biotypes of the predator exist cannot be upheld in this study, but could be verified with other studies suggested below. Guntrip *et al*. (1996), reported significant biological differences between LGB populations from Mexico and Costa Rica, but this cannot be said of *T. nigrescens* from the two countries. One cannot however, rule out the possible existence of strain of *T. nigrescens* along the entire geographical expanse of the pest. Although the observations on the pest's temperature and humidity preference were not conclusive, difference in fitness especially under marginal temperature levels (21 °C and 30 – 33 °C) was observed. It would be important to study the effects of these factors on the developmental and life table characteristics of the pest.

Constant temperature studies provide useful means of modelling populations and predicting the thermal requirements in their development. Such studies have provided useful data for pest management decisions such as pest predictions and timing of control interventions (e.g. Jaramillo *et al*., 2009). These studies are especially useful when variations are distinct, but adjustment to these conditions may mask inters train differences. Also, nature does not provide such constant conditions. Thus the most important effects of the environment would be those extremes that elicit migration or just cause a significant increase in mortality, epizootics, etc. Stimulus mediated gene expression studies would provide a more precise means of understanding the reaction of such extremes on the pest and natural enemy populations (e.g. Fadamiro and Wyatt, 1995; Hodges *et al*., 2003). Heat shock proteins and heat shock factors are molecular chaperons known to mediate such responses. The quantitative expression of this family of genes open a molecular estimate of the possible stress tolerance of living organisms (Malmendal *et al*., 2005) and could provide a new frontier towards understanding possible differences in the adaptability of *T. nigrescens* populations.

This study established the existence of two mitochondrial lineages of the predator associated with geographical zones. The variations associated with such ancestry may not be apparent now, but suggest ancient (and possibly present) genetic differentiation mediated by strong ecological fragmentation. The utilization of this variability across the natural range of the pest may lead to the eventual recovery of better adapted populations. Only two populations of *T. nigrescens* from limited geographical and temporal isolation efforts were released separately in Africa (Meikle *et al*., 2003; Hill *et al*., 2003; Schneider *et al*., 2004). Eventual culturing, quarantine and release possibly reduced the variability and adaptability further. There is need to exploit the potential genetic diversity of *T. nigrescens* and other associated natural enemies for the control of the LGB. A potentially rich source of such diversity would be the contact hybrid zone from central Mexico to northern Honduras. Due to topographical fragmentation, it is possible that several locally adapted subpopulations exist in this zone. Latitudinal similarities with most of Africa also make populations from this area potentially better adapted to local conditions than more subtropical populations from Mexico. In fact the

Costa Rica population that established in West Africa shared similar latitudinal origin with the area of release.

Significant genetic changes in *T. nigrescens* populations were observed in this study representing a period of only 300 generations of laboratory culturing. In nature, this period is too short to engender significant evolution (Lee, 2002). However, laboratory rearing exposes cultures to stochastic and random effects of genetic drift, bottlenecks, modified adaptive selection pressures and fragmentation of populations. Some of these changes may result in loss of fitness similar to the classical screw worm rearing case (Bellows *et al*., 1999). Diploid organisms are a lot more sensitive to such changes (Roderick and Navajas, 2003). High mortality following laboratory release, ecological fragmentation and difficulty to find mates after release pose further stringent bottlenecks in the field. The microsatellite markers developed in this study will be useful for monitoring the rearing, establishment, dispersal and population changes in the new released *T. nigrescens* populations. Consequently, timely intervention and informed decision-making in biological control through the management of genetic diversity would be possible.

Clinal variation along the geographical range exists and may be associated with distinct fitness and predatory characteristics. Genetic recombination would useful in harnessing this diversity for sustainable biological control. Strain interbreeding or joint releases and molecular monitoring may support this approach. *Teretrius nigrescens* is a well adapted predator of the LGB able to effect better control in the wild or when released early. Although *T. nigrescens* reduces grain damage and limits LGB population growth, the damage observed on maize is still much higher than economically tolerable levels. Integrated approaches to LGB control with incorporating management of grain harvesting and processing, storage, monitoring and forecasting, biological, physical and chemical control provide a more pragmatic approach. *Tetetrius nigrescens* would be used in controlling populations off-season and off farm. Collaborative activities to achieve cost effectiveness in fresh recoveries of more diverse populations would enable the utilisation of the full genetic potential of the predator.

Conclusion and recommendations

The initial assumption of this study visualised the success of *T. nigrescens* in Africa as a direct product of ecological suitability of the strains of the predator released. The variation in the species was thought to be distinct, perhaps comparable to well characterised biotypes. This study has shown that while genetic diversity exists between different geographic populations of this species, that variation may be graded and closely tied to local ecological conditions including barriers to migration and population interactions. Also, the methods used in field isolation, rearing and releases of the predator had a role to play in the success of biological control. The predator established in both releases, but effective control required that it locates most breeding populations of the pest. This might have been more difficult in fragmented landscapes, marginal regions and areas where release sizes were small (like in Kenya) compared to west Africa, where propagule pressure was much higher. Published results from West Africa reveal that *T. nigrescens* plays a role in the integrated control of the LGB. This study therefore recommends steps to increase the utility of the full genetic diversity of *T. nigrescens* in the integrated sustainable control of the LGB in other parts of Africa.

Recommendations

- Greater sampling and characterisation of the populations of the larger grain borer across its natural range (Southern USA to northern Columbia/Peru) should be done and compared with the invasive populations in Africa, to locate the critical sources of the African invasion.
- Characterisation of *T. nigrescens* populations from more sites in its native range may give a clearer picture of the extent of genetic diversity there, and perhaps, reveal more efficacious strains.
- Genetic studies and ecological modelling above could be used to predict the regional ecological suitability of these species in Africa and to guide new releases and monitoring.
- A direct study of the expression of stress-related genes and proteins such as heat shock factors, heat shock protein genes may directly reveal the adaptability of the strains to changing climatic conditions. This data could also guide laboratory breeding of laboratory strains for release.

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APPENDIX A: Variation in cloned sequences of *Teretrius nigrescens* internal transcribed spacer sequences (ITS1, ITS2) with flanking and intervening ribosomal RNA gene sequences.

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                      18S (SSU)                         ITS 1 →
hA  GGAAGTAAAA GTCGTAACAA GGTTTCCGTA GGTGAACCTG CGGAAGGATC ATTAACGTTT TTCTCCGCCG CCCTGCTGGT GCGCGCGCGA GAAACAACGC CAACTTATCG TCACGGGACG 120
hB  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... A......... .......... 120
hC  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 120
hD  .......... .......... .......... .......... .......... .........C .......... .......... .......... .......... A......... .......... 120
hE  .......... .......... .......... ..C....... .......... .......... .......... .......... .......... .......... .......... .......... 120
hF  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 120
hG  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 120
hH  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 120
hI  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 120
hJ  .......... .......... ....C..... .......... .......... .......... .......... .......... .......... ...T...... .......... .......... 120
hK  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 120
hL  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... A......... .......... 120

                                                               ITS 1
hA  CCGTGCTCAG GTTTACCAGC GAGTGCCGCG AAGATCGGCA CACGCGGTCC CCGGCGGCCC GAAGACACCT TCTCTCGAAC GCACGACGCC AGCTTGGCGT GGTGGCTCGA GCGGCAGCAG 240
hB  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 240
hC  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 240
hD  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 240
hE  .......... .......... .......... .......... .......... .......... .......... .......... ....A..... .......... .......... .......... 240
hF  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 240
hG  .......... .......... .......... .......... .G........ ......T... .......... .......... .......... .......... .......... .......... 240
hH  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 240
hI  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 240
hJ  .......... .......... .......... ..A....... .......... .......... .......... .......... .......... .......... .......... .......... 240
hK  .......... .......... .......... .......... .G........ ......T... .......... .......... .......... .......... .......... .......... 240
hL  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 240

                                                               ITS 1
hA  CGCGAGAACG CGAACAGAAG AAAGACCCCC GTCGACCGTG CTTCGCGTGA CACGCCGAGC GCGTG--GTC CGCCCGGACG GAGTGATTCG TCTTTCTCGT TCGTCTCGTT CTGCTCCTTC 358
hB  .......... .......... .......... .......... .......... .......... .....--... .......... .......... .......... .......... .......... 358
hC  .......... .......... .......... .......... .......... .......... .....--... .......... .......... .......... .......... .......... 358
hD  .......... .......... .......... .......... .......... .......... .....--... .......... .......... .......... .......... .......... 358
hE  .......... .......... .......... .......... .......... .......... .....--... .......... .......... .......... .......... .......... 358
hF  .......... .......... .......... .......... .......... .......... .....--... .......... .......... .......... .......... .......... 358
hG  .......... .......... .......... .......... .......... .......... .....--... .......... .......... .......... .......... .......... 358
hH  .......... .......... .......... .......... .......... .......... .....--... .......... .......... .......... .......... .......... 358
hI  .......... .......... .......... .......... .......... .......... .....TG.C. .C......GA .......... .......... .......... .......... 360
hJ  .......... .......... .......... .......... .......... .......... .....--... .......... .......... .......... .......... ........C. 358
hK  .......... .......... .......... .......... .......... .......... .....--... .......... .......... .......... .......... .......... 358
hL  .......... .......... .......... .......... .......... .......... .....--... .......... .......... .......... .......... .......... 358
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$(GT)_5$ (low variability) $(ATTCGTCTT)_5$ (non variable)

ITS 1

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hA  GCGTCATACG CCTCGCACAG GTACACGCAC ACTCTCGAAC GCAGTGCACG TGCGCCAGCT CGAGGACACC CCGCCGTCGC CCGAGATATT TCGCCAGACG CGCGCACCCT TTCCCGCCGC  478
hB  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  478
hC  .......... ...T...... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  478
hD  .......... .......... .C........ .......... .......... .......... .......... .......... .........A .T........ .......... ..........  478
hE  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  478
hF  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  478
hG  .......... .......... .......... .......... .......... ........T. .......... .......... .......... .......... .......... ..........  478
hH  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  478
hI  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  480
hJ  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  478
hK  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  478
hL  .......... .......... .C........ .......... .......... .......... .......... .......... .........A .T........ .......... ..........  478
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ITS 1

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hA  ATGTGTTTCT TTCGCAGCGG CAGCGGATCG GCGTGTCGTC GCTGTCGGCC CGGACGATGT GCGTGCGTGT TTTGTCGTCC TCGCCGGCGC ACACGTGCGT GCGCTCCGAC GTGTCCGCGT  598
hB  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  598
hC  .......... .......... .......... .......... .T........ .......... .......... .......... .......... .......... .......... ..........  598
hD  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  598
hE  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  598
hF  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  598
hG  .......... .......... ........T. .......... .T........ ...G...... .......... .......... .......... .......... .......... ..........  598
hH  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  598
hI  .......... .......... .......... .......... .......... .......... .......... .......... .......... G......... .......... ..........  600
hJ  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  598
hK  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  598
hL  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  598
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ITS 1

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hA  TTCGCTTGCG AAAAAACGAG ACGCGTCGGT CGCGCGCCCG TGCAATTTTT TTTTT-AATT GACACAAGTA CAGAACTCGC TTCCGATGCG TTGCGAAAAA CTGTCGTTGA AAAGATTACC  717
hB  .......... .......... .......... .......... .......... .....-.... .......... .......... .......... .......... .......... ..........  717
hC  .......... ..T....... .......... .......... .......... ....--.... .......... .......... .......... .......... .......... ..........  716
hD  .......... .......... .......... .......... .......... .....-.... .......... .......... .......... .......... .......... ..........  717
hE  .......... .......... .......... .......... .......... ....--.... .......... .......... .......... .......... .......... ..........  716
hF  .......... .......... .......... .......... .......... ....--.... .......... .......... .......... .......... .......... ..........  716
hG  .......... .......... .......... .......... .......... .....-.... .......... .......... .......... .......... .......... ..........  717
hH  .......... .......... .......... .......... .......... ....--.... .......... .......... .......... .......... .......... ..........  716
hI  .......... .......... .......... .......... .......... ....--.... .......... .......... .......... .......... .......... ..........  718
hJ  .......... .......... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........  718
hK  .......... .......... .......... .......... .......... .....T.... .......... .......... .......... .......... .......... ..........  718
hL  .......... .......... .......... .......... .......... .....-.... .......... .......... .......... .......... .......... ..........  717
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$(T)_n$ variable

199

5.8S

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hA  CTGAACGGTG GATCACTTGG CTCGTGGGTC GATGAAGAAC GCAGCTAATT GCGCGTCTAC TTGTGAACTG CAGGACACAT GAACATCGAC ATTTCGAACG CACATTGCGG TCCTCGGACG  837
hB  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  837
hC  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  836
hD  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  837
hE  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  836
hF  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  836
hG  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  837
hH  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  836
hI  .......... .......... .......... .......... .......... .......... .......... .........A .......... .......... .......... ..........  838
hJ  .......... ........A. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  838
hK  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  838
hL  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  837
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5.8S ITS 2

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hA  TCTCGTTCCT GGACCACGTC TGTCTGAGGG TCGTCCTCGT ATCAAAATAG CTCCGTGTCT CGCGCACGGG GATTCTTGGG GTCTCGAAGG TCCGTCCGAC CGACGTGCCC TTAAAACACG  957
hB  .......... .......... .......... .......... ....G..... .......... .......... .......... .......... .......... .......... ..........  957
hC  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  956
hD  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  957
hE  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  956
hF  .......... .......... .......... .......... .......... .......C.. .......... .......... .......... .......... .......... ..........  956
hG  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  957
hH  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  956
hI  C......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  958
hJ  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  958
hK  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  958
hL  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  957
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ITS 2

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hA  CGCGCAACGC CGACAGACGT TGCGTGCGTC CTCGCGACGG AACGATAGCC TGCGTCGTTC GTTCTCGTTC GATCGCTAGG CCGCCGTCGC GTGTCGATAC GCGCCCCCCG CGAGCGCGCC  1077
hB  ......G... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1077
hC  ......G... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1076
hD  ......G... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1077
hE  ......G... .......... .......... .......... .......... .......... ...A...... .......... .......... .......... .......... ..........  1076
hF  ......G... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1076
hG  .......... .......... .......... ......G... .......... .......... .......... .......... .......... .......... .......... ..........  1077
hH  ......G... .......... .......... .......... .......... .......... ...A...... .......... .......... .......... .......... ..........  1076
hI  ......G... .......... .......... .......... .......... .......... ...A...... .......... .......... .......... .......... ..........  1078
hJ  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1078
hK  .......... .......... .......... .......... .......... .......... .......... ..C....... .......... .......... .......... ..........  1078
hL  ......G... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1077
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$(TCGTTCGATC)_n$ not variable

ITS 2

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                                            200

hA   GACGCTCTCA CGTGTCCGTG TTGCCGTGCG GCCTTTACGG TCCGGCCCTG CGACGTGAAG CGATCGGTGT CTGAGCGTTC GCGTGCGAGT GCCGTCGTAT CGTCAGCGTT ACGTACAACA  1197
hB   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1197
hC   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1196
hD   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1197
hE   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1196
hF   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......A.. ......G...  1196
hG   .......... .......... .......... .......... .......... .......... .......A.. .......... .......... .......... .......A.. ..........  1197
hH   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1196
hI   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1198
hJ   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1198
hK   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1198
hL   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1197

                                                      ITS 2      28S (LSU)
                                                 <-----------

hA   ACAA---TAT TATTATTATT ATTATTTAAC AACAATA--- ------TTAT TTTATTGTAC GCATAAAACG CGACCTCAGA TCAGGCGAGA TCACCCGCTG AATTTAAGCA TATCAATAAG  1305
hB   ....CAA... .......T-- ---------- ---....GTA ATA---C.G. ..G....... .......... T......... .......... .......... .......... ..........  1299
hC   ....---... .......T-- ---------- ---....ATG ATA---C... ..G....... .......... .......... .......... .......... .......... ..........  1295
hD   ....CAA.G. .......T-- ---------- ---....ATA ATA---C.G. ..G....... .......... .......... .......... .......... .......... ..........  1299
hE   ....---... .......T-- -------... .......ATA ------.... .......... .......... .......... .......... .......... .......... ..........  1298
hF   ....---... .......T-- -------... ..---..ATA ------.... .......... ...A...... .......... .......... .......... .......... ..........  1295
hG   ....---... .......T-- -------... ..---..ATA ------.... .......... .......... .......... .......... .......... .......... ..........  1296
hH   ....---... .......T-- -------... .......ATA ------.... .......... .......... .......... .......... .......... .......... ..........  1298
hI   ....---... .......T-- -------... .......ATA ------.... .......... .......... .......... .......... .......... .......... ..........  1300
hJ   ....---... .......T-- -------... .......ATA ------.... .......... .......... .......... .......... .......... .......... ..........  1300
hK   ....---... .......T-- ---------- ---....ATA ATAATACC.. ..G....... .......... .......... .......... .......... .......... ..........  1300
hL   ....CAA... .......T-- ---------- ---....ATA ATA---C.G. ..G....... .......... .......... .......... .......... .......... ..........  1299
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(TAT)$_n$·(CAA)$_n$· (TAT)$_n$ very variable compound repeat

Sequenced ≈ 1300 bp region of the ITS 1 and ITS 2 from *T. nigrescens* with intervening 5.8S and flanking 18S and 28S rRNA sequences. The coding regions of the first sequence (hA) are underlined and labeled with the names based on sequences from *Ostrinia* sp. and *Tetranynchus* sp.; repeat regions are underlined. Below each batch of sequences, repeat motif indicated and the level of variability observed from 221 cloned sequences from *T. nigrescens*.