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Article

Population Genetic Structure of the Bean Leaf Beetle *Ootheca mutabilis* (Coleoptera: Chrysomelidae) in Uganda

Dalton Kanyesigye[1,5], Vincent Pius Alibu[2], Wee Tek Tay[4], Polycarp Nalela[1], Pamela Paparu[1], Samuel Olaboro[1], Stanley Tamusange Nkalubo[1], Ismail Siraj Kayondo[6], Gonçalo Silva[3], Susan Elizabeth Seal[3], Michael Hilary Otim[1,*]

1 National Agricultural Research Organization (NARO), National Crops Resources Research Institute (NaCRRI), P.O Box 7084, Kampala, Uganda; kanyesigyedalton@gmail.com (D.K); nalelapolycarp@gmail.com (P.N); pamela.paparu@gmail.com (P.P); olasamuel94@gmail.com (S.O); tamusange@gmail.com (S.T.N); motim9405@gmail.com (M.H.O)
2 College of Natural Sciences (CoNAS) Makerere University, Uganda; vpalibu@yahoo.com (V.P.A)
3 Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK; G.Silva@greenwich.ac.uk (G.S), S.E.Seal@greenwich.ac.uk (S.E.S)
4 Commonwealth Scientific and Industrial Research Organisation, Canberra, ACT 2601, Australia; Weetek.Tay@csiro.au (W.T.T)
5 College of Veterinary Medicine, Animal Resources and Biosecurity (CoVAB), Makerere University, Uganda; (D.K)
6 International Institute of Tropical Agriculture, PMB 5320, Oyo Rd., Ibadan, Nigeria; kayondowork@gmail.com (ISK)
* Correspondence: motim9405@gmail.com, motim@naro.go.ug;

Simple Summary: Bean leaf beetle (*Ootheca mutabilis*) has recently become an important bean pest in Uganda, causing devastating crop losses. There is limited information on their population genetic structure despite their importance. We developed microsatellites, which we used in combination with the partial mitochondrial cytochrome oxidase subunit I gene sequences to analyze the spatial population genetic structure of 87 *O. mutabilis* samples from five populations. We developed a suite of five highly polymorphic microsatellites (4-21 alleles, heterozygosity 0.59-0.84, polymorphic information content 50.13-83.14%). Microsatellite analysis resulted in 89% of genetic variation occurring within individuals, 9% among individuals and 2% among populations. Genetic differentiation was low but significant for microsatellites and insignificant for mitochondrial cytochrome oxidase subunit I gene partial sequences while gene flow was high. No isolation by distance was found between geographical and genetic distances. Bayesian clustering identified signature of admixture that suggests genetic contributions from two ancestral genetic lineages, and the median-joining haplotype network showed low differentiation of many different haplotypes from the most common haplotype. Low genetic differentiation and high gene flow indicate unrestricted movements between populations. This information will contribute to the design of *O. mutabilis* control strategies.

Abstract: Bean leaf beetle (BLB) (*Ootheca mutabilis*) has emerged as an important bean pest in Uganda, leading to devastating crop losses. There is limited information on the population genetic structure of BLB despite their importance. In this study, novel microsatellite markers and the partial mitochondrial cytochrome oxidase subunit I (mt*COI*) gene sequences were used to analyze the spatial population genetic structure, genetic differentiation, gene flow and haplotype diversity of 87 *O. mutabilis* samples from five populations. We identified 19,356 simple sequence repeats (SSRs) (mono, di, tri, tetra, penta, and hexa-nucleotides) of which 81 di, tri and tetra-nucleotides were selected for primer synthesis. Five highly polymorphic SSR markers (4-21 alleles, heterozygosity 0.59-0.84, polymorphic information content (PIC) 50.13-83.14%) were used for this study. Analyses of the five *O. mutabilis* populations with these five novel SSRs found 89% of genetic variation occurring within individuals, 9% among individuals and 2% among populations. Genetic differentiation was low but significant for SSR and insignificant for mt*COI* partial sequence data while gene flow was high across the populations. There was no evidence of isolation by distance between geographical and genetic distances. Bayesian clustering identified signature of admixture that suggests genetic contributions from two ancestral genetic lineages, and the median-joining haplotype network showed low differentiation of many different haplotypes from the most common haplo-

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type. Low genetic differentiation and high gene flow indicates unrestricted migrations between populations. This information will contribute to the design of BLB control strategies.

Keywords: Genetic differentiation; leaf beetle; mitochondrial DNA; microsatellites; haplotype; gene flow

1. Introduction

The bean leaf beetle (BLB) (*Ootheca mutabilis* (Coleoptera: Chrysomelidae)) is an economically important pest of common bean (*Phaseolus vulgaris* L.) in East and Southern Africa [34]. The larvae feed on and destroy the root tissue while adults voraciously feed on and skeletonize leaves, flowers and immature pods [18]. Complete crop losses may occur during heavy infestations [2,34]. Bean leaf beetles are also associated with virus spread in cowpeas [1,34,46]. *Ootheca mutabilis*, is the most prevalent (70.3%) *Ootheca* species in all agro-ecological zones of Uganda [17], although it was reported as mainly found in lowlands [34]. However, its abundance and damage on common bean in Uganda varies across locations and seasons [17]. Furthermore, *O. mutabilis* exists in different colour forms and is often indistinguishable from other *Ootheca* species such as *O. proteus*, *O. bennigseni* and *O. orientalis* [26]. The morphological identification records have undergone revision whereby some previously described *Ootheca* species have been redescribed and transferred to other species [26]. Currently, the only reliable morphological identification method is through dissection and examination of the male genitalia [26], a method that excludes the species-level identification of females and other growth stages. Therefore, there is a need to use molecular diagnostic methods, with the use of partial mitochondrial DNA cytochrome c oxidase subunit I (mt*COI*) sequences being a favoured method globally and that has been used successfully in our Ugandan laboratories for other agricultural pests (for example, [39,38,36]. The spatial population genetic structure of *O. mutabilis* has not been determined. An understanding of BLB population structure, particularly whether discrete and genetically unique subpopulations exist, is therefore necessary for the development of optimal management strategies for area-wide management of these destructive agricultural pests. Microsatellite genetic markers, also known as simple sequence repeat (SSR) markers, are comprised of tandem repeated nucleotide motifs of 1-6 base pairs (bp). They have been used widely in evolutionary genetic studies [7,50,35] to infer life history including mating behavior [7,56,55], mating frequencies [19], gene flow patterns [55], dispersal [42,30], range shifting [42], and host-shifting [43]. The advantages of using microsatellites in genetic diversity studies is that they are relatively abundant, co-dominant, ubiquitous and extensively polymorphic [35]. However, it can be difficult to isolate them in certain insect orders [60,54,49] due to association with mobile elements [54,15]. Microsatellites have been applied in a number of insects including European stag beetle [33] and red flour beetle [8]. We studied the population genetic structure of *O. mutabilis* using a suite of five novel microsatellite DNA markers. We used the mt*COI* partial gene to barcode the morphologically indistinguishable samples, and used the same DNA sequences to analyse the *O. mutabilis* haplotypes, haplotype diversity and haplotype distribution.

2. Materials and Methods

2.1 Sample collection

We collected BLBs from 17 districts (Fig. 1 and (Table 1) in five bean production agro-ecological zones (Table 1) during both rainy seasons of 2016, 2017 and 2018. Collection involved sampling adult BLBs in farmers' common bean fields. We selected at least one district per agro-ecological zone from which, two sub-counties were selected. From

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each sub-county, we collected between 10-100 beetles from 10 gardens. Each beetle sample was immediately placed in a screw-capped 2 ml vial containing 95% ethanol and the ethanol was changed at least twice at daily intervals to avoid DNA degradation due to adult beetle fluids. Samples were transported to the National Crops Resources Research Institute (NaCRRI), Namulonge in Wakiso district and kept at room temperature before further analyses.

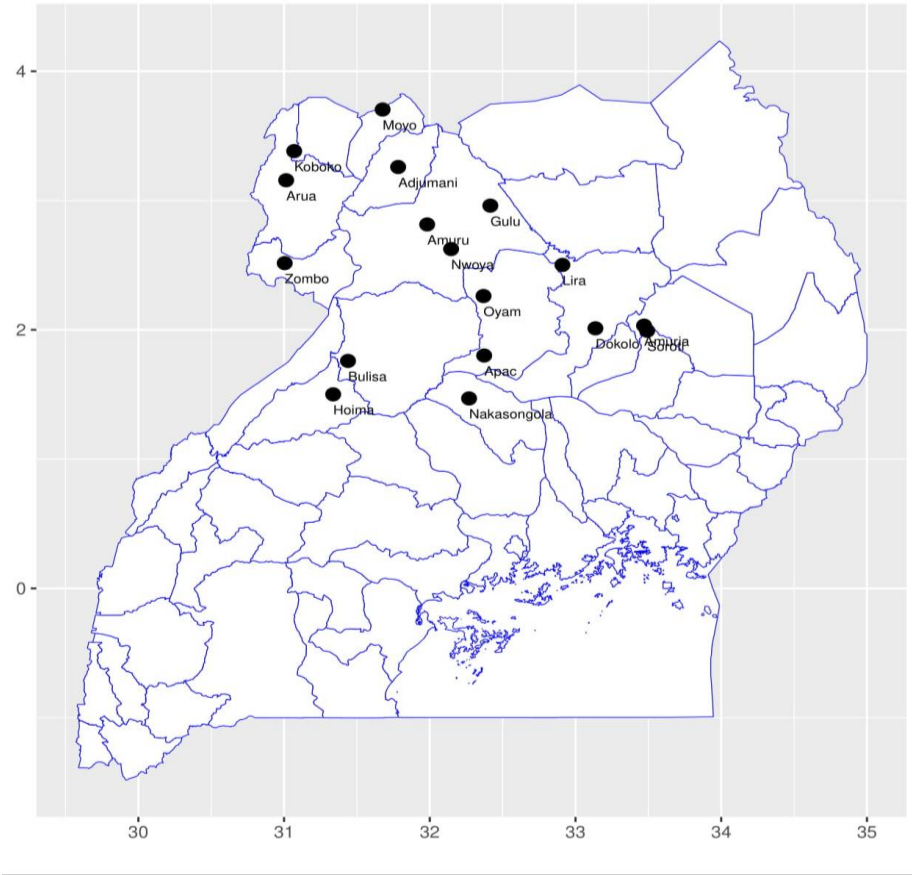

Figure 1. Districts in Uganda showing collection locations for bean leaf beetle samples used in this study.

Table 1. Agro-ecological zones and the districts where bean leaf beetle samples *mutabilis* samples were collected.

Population Code	Agro-ecological zone	Districts
A	Northern moist farmlands	Dokolo, Lira, Oyam, Apac, Amuru, Gulu and Nwoya
B	Southern and Eastern Lake Kyoga basin	Amuria and Soroti
C	Western mid altitude farmlands	Nakasongola
D	Central wooden savannah	Bulisa and Hoima
E	North-western farmlands	Adjumani, Zombo, Koboko, Moyo and Arua

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2.2 Identification of bean leaf beetles

Representatives of BLB samples were morphologically identified at the Universitätkoblenz-Landau Institut für Integrierte Naturwissenschaften Abteilung Biologie Universitätsstraße 156070 Koblenz, Germany. The bean leaf beetle samples used in population genetic structure were initially selected for molecular analysis based on colour patterns of elytra, head, thorax, abdomen and legs as reported by [26]. On the basis of appearance, we selected 99 samples denoted as: M1 (i.e., *O. mutabilis* with elytra upper half black and lower half yellowish) (21), M2 (i.e., *O. mutabilis* with black elytra) (39), and M3 (i.e., *O. mutabilis* with brownish elytra) (39) (Fig. 2). This separation based on colour was to enable assessments of possible genetic differences among morphotypes of *O. mutabilis*.

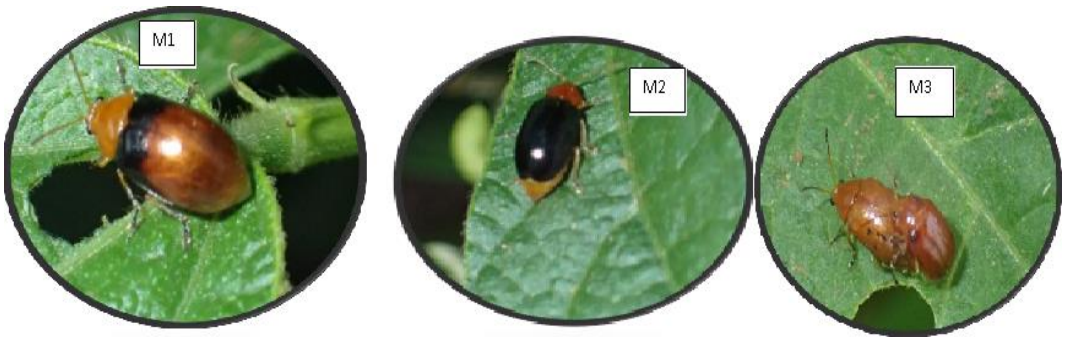

Figure 2: Different colour-morphs of *Ootheca mutabilis.* M1: *O. mutabilis* with elytra upper half black and lower half yellowish; M2: *O. mutabilis* with black elytra; M3: *O. mutabilis* with brown elytra.

2.3 DNA isolation and quantification

We used the DNeasy Blood and Tissue Kit (www.qiagen.com) for DNA isolation following the manufacturer's protocol. After isolation, each DNA sample was quantified using an Agilent Nanodrop 2000 spectrophotometer (www.fishersci.com) and the quality checked using 1% agarose gel electrophoresis.

All samples selected based on colour were barcoded using the mt*COI* partial gene primers to confirm their identity. The PCR primers used were; BLB-LCO: 5'-GGTCAACAAATCATAAAGATATTGG-3', BLB-HCO: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' that amplify a 710 bp fragment (Otim *et al*., unpublished). Each reaction was performed in 25 µl volume containing 1 µl of template DNA, 1 µl each of 10 pmol/µL of primer, 2.5 µl of 10X DreamTaq Green buffer, 0.5 µl of dNTP (10 mM), 0.25 µl (1.25 units) of *Taq* DNA polymerase (5 U/µl), 2.5 µl 5% Tween20, 16.25 µl of nuclease free water. The PCR conditions were: initial 1 cycle of denaturation for 2 min at 95 °C, 35 cycles of 20 s at 95 °C, 30 s at 52 °C annealing temperature, 1 min at 72 °C and a final extension cycle at 72 °C for 10 min after which, reactions were held at 4 °C. The amplification success for all samples was checked on 1.3% w/v agarose in TAE buffer gels and then stained with ethidium bromide as described above. Sequences generated from the mt*COI* PCR products were processed using Pregap4 and Gap4 [51], and compared with sequences of *O. mutabilis* (KY574530.1, KY574526.1, KY574527.1) and *O. proteus* (KY574525.1, KY574524.1, KY574523.1, KY574522.1) downloaded from NCBI. A pairwise distance matrix table was generated for *O. mutabilis*, *O. proteus* and *Longitarsus tabidus* sequences (Table 2) using the uncorrected pair-wise nucleotide distance (*p*-dist) model in MEGA7. A neighbour joining (NJ) phylogenetic tree of different *O. mutabilis* haplotypes was constructed. A sequence of leaf beetle *L. tabidus* (KU917773.1) was downloaded from NCBI to use as outgroup during phylogenetic tree construction. The sequences were trimmed post-alignment and then imported to Gene-

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ious v10.2 [24] for further analyses. Multiple sequence alignment and consensus sequence building were performed using Multiple Sequence Comparison by Log-Expectation (MUSCLE). Then a NJ phylogenetic tree constructed using Jukes-Cantor genetic distance model [23] with 70% support threshold in bootstrap re-sampling method using 1,000 number of replicates.

Table 2. Pairwise distances of *Ootheca proteus* (GenBank KY574521.1, KY574525.1) and *O. mutabilis* (GenBank KY574527.1, KY574530.1) downloaded from NCBI, *O. proteus* (M3094_C, M3095_C) and *O. mutabilis* (GenBank MW278873.1) found in the study and *L. tabidus* (outgroup; KU917773.1). The number of base substitutions per site from between sequences are shown below diagonal and standard error estimates are shown above the diagonal.

KU917773.1_*L. tabidus*		0.012	0.012	0.012	0.012	0.012	0.012	0.012
KY574521.1_*O. proteus*	0.120		0.001	0.010	0.010	0.001	0.000	0.011
KY574525.1_*O. proteus*	0.119	0.002		0.010	0.010	0.002	0.001	0.010
KY574527.1_*O. mutabilis*	0.129	0.085	0.084		0.004	0.011	0.010	0.003
KY574530.1_*O. mutabilis*	0.123	0.078	0.076	0.012		0.010	0.010	0.004
M3094_C_*O. proteus*	0.122	0.002	0.003	0.087	0.079		0.001	0.011
M3095_C_*O. proteus*	0.120	0.000	0.002	0.085	0.078	0.002		0.011
MW278873.1_*O. mutabilis*_M3-Hap1	0.131	0.087	0.085	0.005	0.011	0.088	0.087	

2.4 Genome sequencing for microsatellite analysis

High throughput sequencing (HTS) was outsourced to GENEWIZ (www.genewiz.com). DNA was extracted from three ethanol-preserved BLBs coded M1, M2, and M3. Whole-genome DNA libraries were constructed and sequenced using the Illumina HiSeq 2500 system, with an insert size of 300-400 bp and 2 x150 paired end reads.

2.5 Quality check and raw read assembly

Before processing raw reads for *de novo* assembly, their quality was first checked using Fast QC v0.11.7 [4]. After QC, raw DNA reads were processed; adaptor sequences trimmed, duplicate sequences removed and then the sequences were *de novo* assembled. Raw read processing and *de novo* assembly were all performed in Geneious v10.2 [24] using default settings (i.e., allow gaps- maximum gaps per read 20%, ignore words repeated more than 1000 times, do not merge variants with coverage over approximately 6, merge homopolymer variants).

2.6 Microsatellite prediction and primer design

Microsatellites in the assembled contigs were identified using Websat [32] followed by primer design using Primer3 [45]. The settings for primer design included primer size range of 18 nucleotides at minimum, 22 at optimum and 27 at maximum. Primer Tm was 57 °C at minimum, 60 °C at optimum and 68 °C at maximum. The primer GC% range was 40 to 80. The product size range was 100-400 bp. After designing the primers, 81 desalted primer pairs were ordered from Macrogen Europe (dna.macrogen.com). Primers were designed for all di-nucleotide, tri-nucleotide and tetra-nucleotide microsatellite loci identified.

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2.7 Blast search of microsatellite sequences in GenBank

Microsatellite sequences were compared with the microsatellite sequences in the NCBI GenBank using BLASTX and BLASTN [52] to determine if they shared similarity with sequences of other insects and the potential retrotransposible elements (e.g., [54].

2.8 Microsatellite DNA marker PCR optimization, polymorphism testing, primer labelling and fragment analysis

Each microsatellite primer pair was first optimized for amplification prior to being tested for polymorphism on eight *O. mutabilis* samples (at least one sample from every population). Each locus was amplified in a 12.5 µl PCR reaction containing 0.5 µl of 50 ng DNA template, 0.5 µl each of 10 pmol/µL of primer, 1.25 µl of 10X DreamTaq green buffer, 0.25 µl of DreamTaq dNTP (10 mM), 0.125 µl of DreamTaq DNA polymerase (5U/µl), 1.25 µl 5% Tween20, and 8.125 µl of nuclease free water. The PCR conditions used were: initial denaturation (4 min 94 °C) followed by 35 amplification cycles (20 s 94 °C, 30 s annealing temperature, 45 s 72 °C) and a final extension (10 min 72 °C). The reactions were later held at 4 °C. Amplicons were run first on 3% w/v agarose in 1x TAE gels and then later tested for polymorphism with 100 bp DNA ladder in 1X TAE buffer for 6 hours at 120 volts on 6% acrylamide gels. Polyacrylamide gels were stained by immersing them in a solution of ethidium bromide (0.5 µg/ml) for at least 20 minutes and destained using distilled water. Fragment sizes were visualized using U-genius gel documentation system (www.syngene.com). Each locus was tested for reproducibility at least twice. After primer optimisation, five primers were selected based on their polymorphism on polyacrylamide gels. These primers were ordered from Macrogen (dna.macrogen-europe.com). Each of the forward primers was either labelled with 6-FAM or HEX fluorescent dyes (dna.macrogen-europe.com) (Table 3). The PCR reactions were performed in single reactions and later, each 6-FAM and HEX PCR products with similar amplicon product size ranges were pooled and run as multiplex. Fragment analysis was performed using Applied Biosystems 3730XL DNA Analyzer (outsourced to Macrogen Europe)

2.9 Genotyping and data scoring

Genotyping was performed using GeneMarker v2.6.3 [21] and alleles scored according to their size. The quality of allele scoring was checked using MICROCHECKER [58]. Polymorphic information content (PIC), observed and expected heterozygosity (H_o, H_e) were calculated using Molkin v3.0 [16].

2.10 Data analysis

2.11 Population genetic structure and differentiation

Measures of mtDNA diversity; number of haplotypes, haplotype diversity, nucleotide diversity and polymorphic sites estimates were calculated using DnaSP v6 [44]. The AMOVA and gene flow estimates from SSR data were performed using GenAlEx v6.503 [40] with 9999 permutations. Geographic structures of *O. mutabilis* populations using SSR data were investigated using the Bayesian approach implemented in STRUCTURE v2.3.4 [41]. STRUCTURE uses a coalescent genetic approach to cluster similar multilocus genotypes into inferred ancestral genetic clusters (K), regardless of an individual's geographical origin. We conducted ten independent runs for each value of K ranging from 1 to 5 using the admixture model. Each run consisted of a burn-in of 50,000 steps followed by 100,000 Markov chain Monte Carlo (MCMC) repetitions. Ten replicates were used for each potential value of K. The STRUCTURE runs were performed using LOCPRIOR command, in which genotypes defined were based on the geographic location of the *O. mutabilis* samples. The true value of K was estimated as described in [12] using the pro-

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gram STRUCTURE HARVESTER [9]. The structure results were visualized using CLUMPAK [25]. *F*st (SSR data) and PhiPT (mt*COI* haplotypes) estimates were calculated to estimate the degree of genetic differentiation over all populations using GenAIEx v6.503 with 9999 permutations

2.12 Haplotype analysis

The mt*COI* haplotype network was inferred based on the median joining network approach implemented in POPART [29] using 2000 bootstraps based on sequence alignment that was exported from DnaSP v6 as a nexus file of haplotypes.

2.13 Isolation by distance (IBD)

The IBD (SSR data) was tested using Mantel test [31] using GenAIEx v6.503 with 9999 permutations.

3 Results

3.1 Quality check of NGS sequences and de novo assembly

A total of 272,853,156 raw reads made up of equal forward and reverse reads were generated. All sequences had a uniform length of 151 bp with 34% GC content. QC results included basic statistics passed, per base sequence quality passed, per tile sequence quality passed, per sequence quality scores passed, per base sequence content passed, and sequence length distribution passed. The sequences were not overrepresented. Assembly of all sequences in the selected part of the reads produced a total of 282,696 contigs.

3.2 SSR prediction and primer design

A total of 19,356 simple sequence repeats consisting of: (i) mononucleotides (14,629, 75.6%), (ii) di-nucleotides (2,780, 14.4%), (iii) tri-nucleotides (1,288, 6.7%), (iv) tetra-nucleotides (352, 1.8%), (v) penta-nucleotides (258, 1.3%), and (vi) hexa-nucleotides (49, 0.3%) were identified from the assembled contigs.

3.3 Microsatellite PCR optimization and polymorphism testing

Of the 81 loci tested, 65 had multiple fragments on agarose gels and were excluded from the analysis. A total of 16 loci were observed as a single band on agarose gels and also appeared polymorphic on 6% acrylamide gels. Of these, 16 polymorphic loci, six were removed because they showed poor PCR amplification (i.e., fuzzy bands in some samples, failed PCR amplification in others). Two of the 10 loci had their primers overlapping the (GT) SSR units and were therefore also excluded. Of the remaining eight loci, we further excluded three loci due to poor reproducibility. Five loci were therefore considered for genotyping (representing a success rate of 6%) and were labelled with fluorescent dyes (Table 2). The numbers of alleles for our SSR markers ranged from 4 to 21 with an average of 11.6 (Table 3).

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Table 3. Characteristics of the five developed microsatellite loci for *Ootheca mutabilis*. NA (Number of alleles)

Locus name	Motif	Size (bp)	Primer sequence 5′ ⟶ 3′	GenBank Accession number	NA	Tm (˚C)	Fluorescent label
BLB2_om1	$(GAT)_2(CAA)_{11}$	343-365	F: TCAACTACCACCATCACAAACC R: CAATGTGGAGCAACTACGTCAT	MT074093	9	58	5′6-FAM
BLB2_om17	$(CTT)_{10}$	368-396	F: CCAATCCGCTTCTCTATATCCA R: GGAGCAATGTTATGCCTGATTT	MT074094	16	57	5′6-FAM
BLB2_om32	$(GACG)_6$	160-195	F: CATATAGCGAAAACCCGAAATC R: AGAAGTACAAGTATGGCCCGAA	MT074096	21	58	5′6-FAM
BLB2_om33	$(ACA)_5.(ACG).(ACA)_{16}$	256-288	F: ATTGAAAGTTGTATCGGTCGCT R: CTTGACATGAAAACGAGATCCA	MT074095	4	58	5′HEX
BLB2_om66	$(AGT)_2(AGC)_7$	337-345	F: CTATGGTCGTTTTCTCCGACAT R: GACGTTTCTTCTCGGTTGTAGC	MT074097	8	60	5′HEX

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3.4 Fragment analysis and allele scoring

The five loci used in this study were all polymorphic with an average PIC of 69.1%. Locus BLB2_om66 had the lowest PIC of 50.1% and BLB2_om33 had the highest PIC of 83.1% (Table 4). A locus was considered to have low polymorphism with a *PIC* of <25%; and high polymorphism with a *PIC* of >50% [48]. The polymorphism levels of the five microsatellite loci used in this study were high, with *PIC* values ranging from 50.1- 83.1%.

Table 4. Diversity indices of the five microsatellite loci developed for *Ootheca mutabilis*. Heterozygosity and polymorphic information content are *He* and PIC, respectively.

Locus name	*He*	PIC
BLBom1	0.75	75.0%
BLBom17	0.65	58.8%
BLBom32	0.80	78.5%
BLBom33	0.84	83.1%
BLBom66	0.59	50.1%
Average	0.73	69.1%

Across all populations, the average PIC based on all loci was 53.59% (Table 5). The average heterozygosity for all loci was 0.73 with the locus BLB2_om33 having the highest heterozygosity of 0.84 and BLB2_om66 having the lowest heterozygosity of 0.59 (Table 4). Observed heterozygosity was higher than expected heterozygosity in all populations ranging from 0.75-0.84 with an average observed heterozygosity of 0.80 (Table 5)]. Loci BLB2_om17 and BLB2_om32 had excess of homozygosity and could indicate potential presence of null alleles or allele drop-out.

Table 5. Gene diversity in the five populations of *Ootheca mutabilis*. Alphabet letters represent populations; (A) Northern moist farmlands (B) Southern and Eastern Lake Kyoga basin (C) Western mid-altitude farmlands, (D) Central wooden savannah and (E) North-western farmlands. Observed heterozygosity (H_o); expected heterozygosity (H_e) and polymorphic information content (*PIC*) are shown.

Population	H_o	H_e	PIC
A	0.82	0.72	56.97%
D	0.80	0.68	51.48%
C	0.75	0.66	47.42%
B	0.84	0.73	57.72%
E	0.78	0.70	54.36%
Average	**0.80**	**0.70**	**53.59%**

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3.5 Population genetic structure, differentiation and gene flow

Analysis of all 99 mt*COI* partial (i.e., 658bp) gene sequences identified two species (*O. mutabilis* and *O. proteus*) of which 87 were *O. mutabilis* and 12 were *O. proteus*. Analysis of the 87 *O. mutabilis* mt*COI* partial gene sequences detected 21 segregating sites (S) with an average of 0.827 nucleotide differences (Kt). We detected 20 haplotypes with an estimated haplotype diversity (H, also known as gene diversity, a measure of the probability of two random alleles being different [37] of 0.50735, and low nucleotide diversity Pi (π) of 0.00126 (i.e., the average number of nucleotide differences per site in pairwise DNA sequence comparison [37]. We detected moderate haplotype diversity between the 20 haplotypes GenBank: MW278873 - MW278892 (Fig. 3).

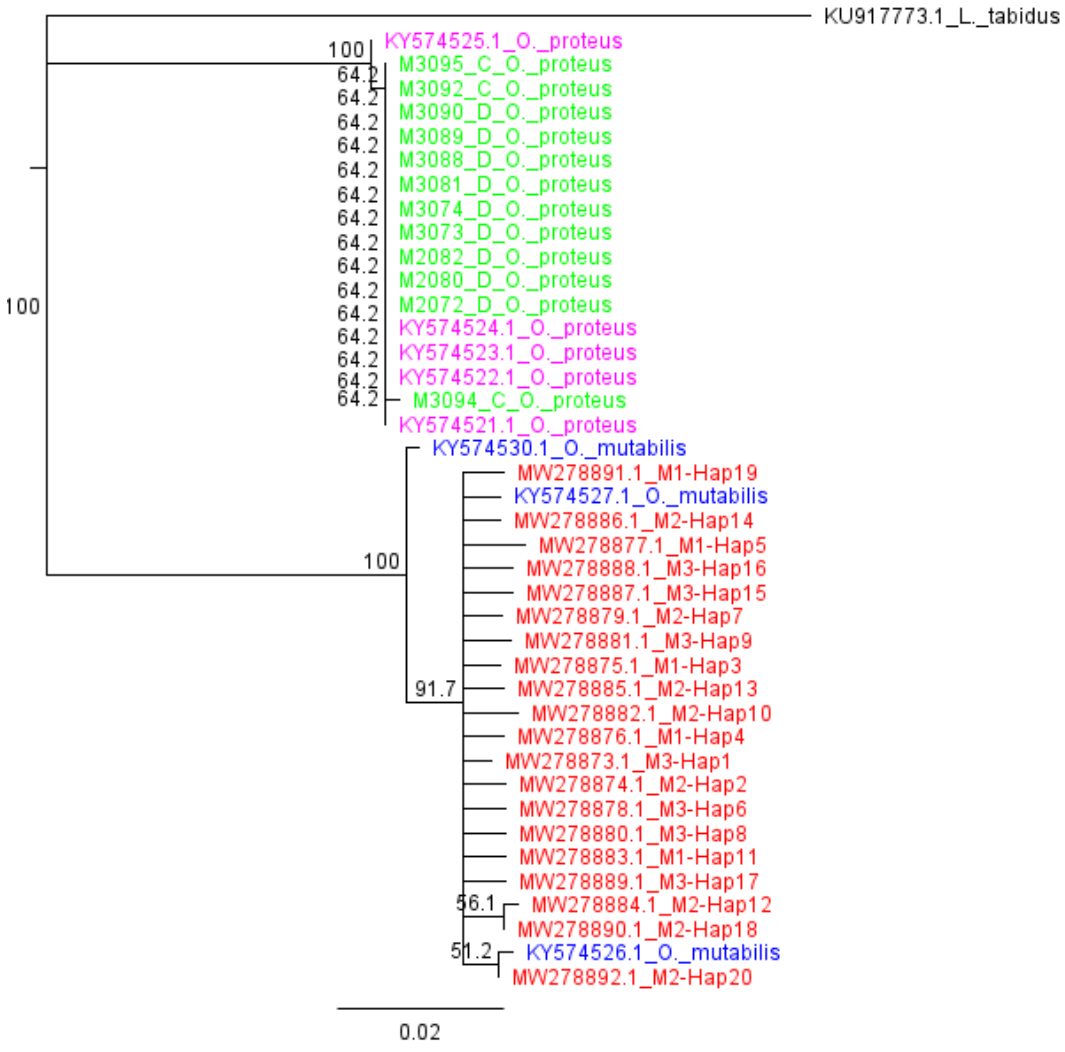

Figure 3. Neighbour Joining phylogenetic tree of the 20 *Ootheca mutabilis* haplotypes (red) found in the study, and reference sequences downloaded from NCBI as follows: *O. mutabilis* (blue), *O. proteus* (pink), *O. proteus* (green, morphologically similar samples to *O. mutabilis* separated after DNA-barcoding) and the outgroup *Longitarsus tabidus*.

Analysis of molecular variance for SSR data revealed that the highest percentage of variation (89%) was within individuals followed by among

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individuals (9%) and finally among populations (2%) (Table 6). Population A contained more samples (40) and therefore, a separate AMOVA was performed by dividing population A into sub-populations based on different districts (Dokolo, Lira, Oyam, Apac, Amuru, Nwoya and Gulu). The result (data not shown) was similar to the results for the whole population.

Table 6. Analysis of Molecular Variance (AMOVA) for the five populations of *Ootheca mutabilis*. Degrees of freedom (Df); sum of squares (SS); mean square (MS); Est.Var (Estimated Variance); Number of migrants per generation (Nm).

Source of variation	Df	SS	MS	Est. Var	Variation	F-statistics	P-Value	Nm
Among Populations	4	14.15	3.537	0.04	2%	0.018	0.003	
Among Individuals	82	190.42	2.322	0.20	9%	0.096	0.0001	
Within Individuals	87	166.50	1.914	1.91	89%	0.113	0.0001	
Total	173	371.07		2.16	100%			13.278

Genetic differentiation (SSR data) of all the populations was measured using the *F*-statistics (i.e., *F*st, *F*is, *F*it) based on microsatellite. Analysis of SSR data over all the populations revealed differentiation at a low but significant level (i.e., *F*st = 0.018, p = 0.0024; *Fis* = 0.096, p = 0.0001; *Fit* = 0.113, p = 0.0001) and insignificant for mt*COI* data PhiPT = 0.0005, *P* (0.429). The gene flow (SSR data) estimate based on an effective number of migrants per generation (Nm) was high, at 13.3 despite the significant *F*st estimate. STRUCTURE analysis [12] based on the SSR markers identified K = 2 as the likely optimal number of ancestral genetic clusters, with all individuals grouped as one dominant cluster (blue colour) and varying degrees of genetic contributions (orange colour) from a second minor genetic cluster (Fig. 4).

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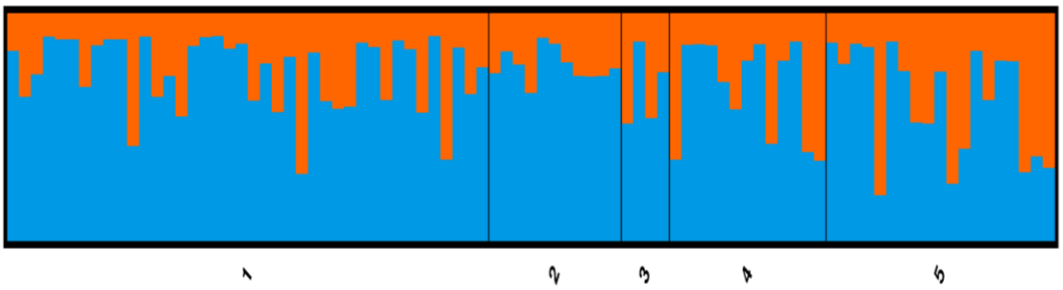
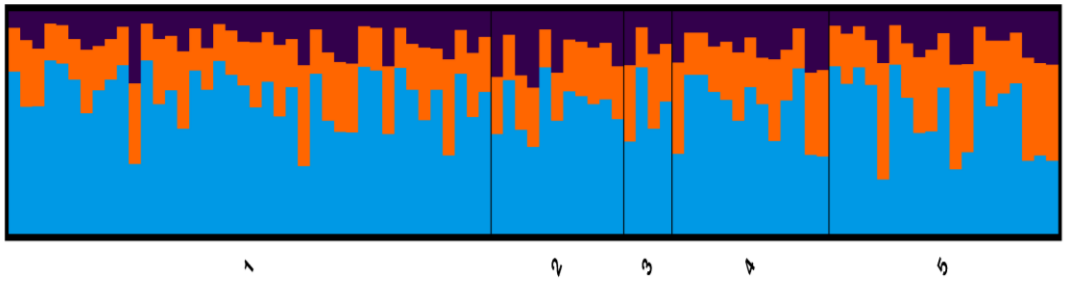
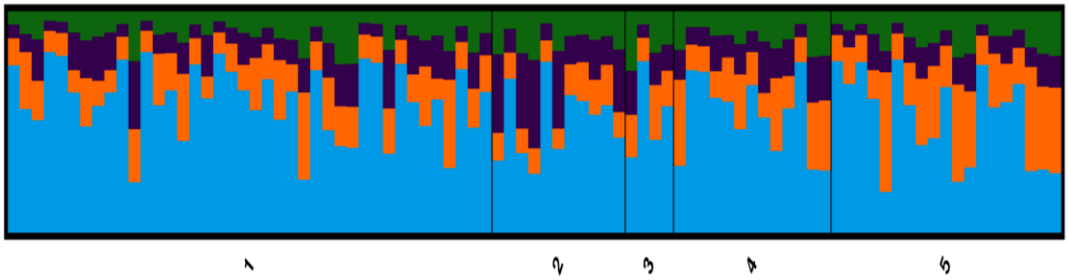
K2 (True value of K)



K3



K4



K5

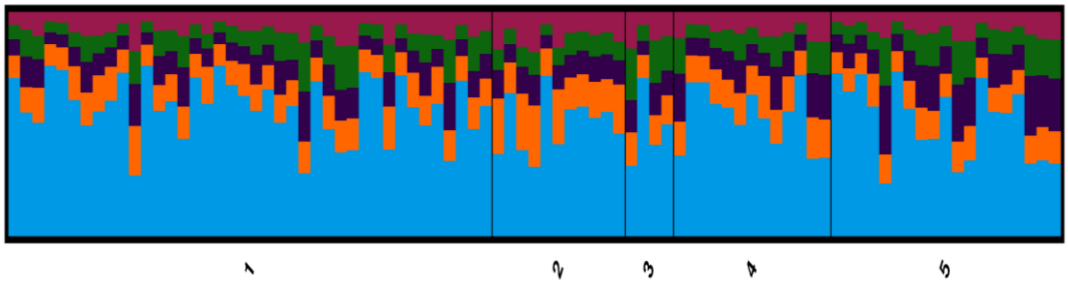


Figure 4. Population structure across five populations of *Ootheca mutabilis* obtained using structure (for microsatellite data) for K2 to K5. Vertical bars represent individuals. Numbers represent populations; (1) Northern moist farmlands (A), (2) Central wooden savannah (D), (3) Western mid-altitude farmlands (C) (4) Southern and Eastern Lake Kyoga basin (B) and (5) North-western farmlands (E).

3.6 mtCOI haplotype network of O. mutabilis

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The median-joining haplotype network (Fig. 5) showed one major haplotype (haplotype 1), which was found in all five populations (A, D, C, B, and E) and in 70.1% (n = 61) of the individuals. The second most common haplotype was haplotype 14, found in 5.75% of individuals (n = 5). Three of the haplotypes (6, 7 and 18) had 2.30% each with two individuals. The remaining 15 haplotypes had n = 1 each (i.e., 1.15% each). Haplotype 15 (i.e., the second most common haplotype) was present in three populations (A, B and E). Haplotypes 6, 7 and 18 with the third-highest frequency (2.30% each) varied in populations although most of the individuals (n = 4) belonged to population 1 (Fig. 5).

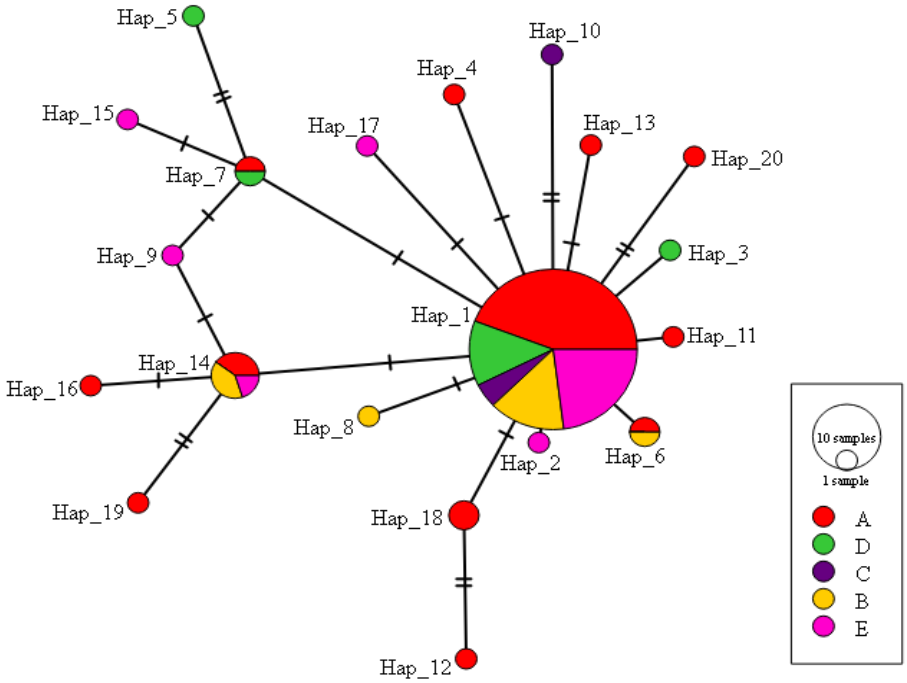

Figure 5. Median-joining haplotype Network showing evolutionary relationships among haplotypes. Each small black stick along a connecting line represents a change of one base pair. Haplotypes are colour coded according to the population (red represents population (A) Northern moist farmlands, green represents population (D) Central wooden savannah, purple represents population (C) Western mid-altitude farmlands, yellow represents population (B) Southern and Eastern Lake Kyoga basin and pink represents population (E) North-western farmlands). Circle sizes correspond to the haplotype numbers.

3.7 Isolation by Distance (IBD)

No evidence of IBD (SSR data) was found in the 87 samples (Mantel test; r = 0.049, p = 0.167). Most of the dissimilarity matrices (genetic distances) had values between 3.0 and 16.0 (Fig. 6) and the geographic distance dissimilarity scatter plots (Geographic distances) showed random dispersion of the matrices for all samples analyzed. The geographic distances (Kms) ranged from 0.0 to 441.9 (Fig. 6).

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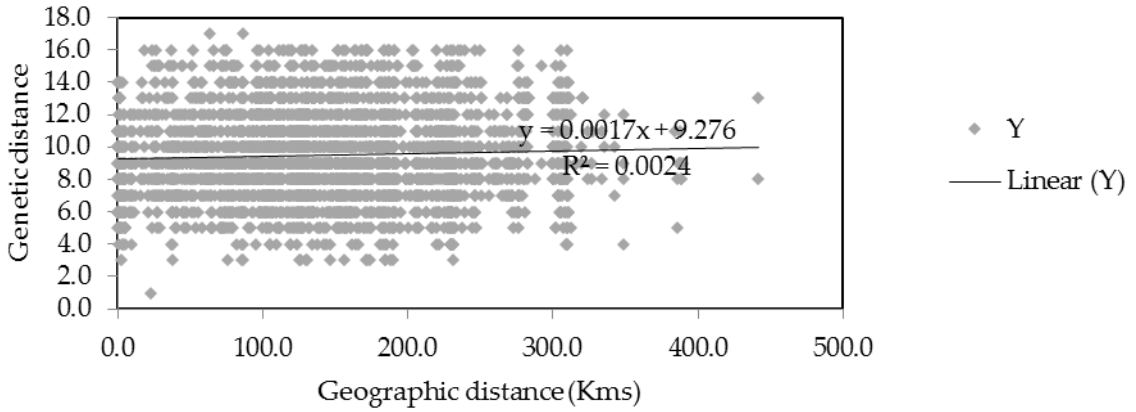

Figure 6. Correlation between geographical and genetic distances of *Ootheca mutabilis* samples.

4. Discussion

We assessed the genetic structure, differentiation and gene flow between five populations of *O. mutabilis* from different agro-ecological zones of Uganda using microsatellites and mt*COI* markers. Based on nuclear DNA markers (SSR data), we found that 89% of genetic variation occurred within individuals, 9% was among individuals, while only 2% was among populations. Genetic differentiation was low but significant for SSR data and insignificant for mt*CO1* data, while gene flow (SSR data) was high across the populations and there was no evidence of isolation by distance (SSR data). With the mt*COI* marker being a maternally inherited marker and the SSR markers being co-dominant, the finding of low but significant population substructure based on SSR markers but not the mt*COI* marker could suggest potential dispersal behavioural differences between *O. mutabilis* males and females, although field marked-recapture experiments would be needed to investigate this further.

The higher level of variation found within individuals than among individuals indicates a high level of interbreeding between the individuals in the sample locations. The F_{IS} and F_{IT} results also support high interbreeding within individuals. This is the first population genetic structure study on *Ootheca* spp. In another leaf beetle *Cerotoma trifurcata* in the mid-western USA, [57] reported similar high variation within samples, while the lowest level of variation was among populations and thus indicated high levels of gene flow. Low levels of variation among populations can be attributed to the presence or absence of geographical barriers to restrict gene flow. The absence of geographical barriers among groups of *C. trifucarta* in mid-western USA t favoured dispersal and reduction in both geographical fragmentation and genetic differentiation [57].

The low but significant *F*st value in our study indicated low genetic differentiation and suggests that migration is occurring between *O. mutabilis* populations including between those separated by large geographic distances. Local and geographically distant migration of *O. mutabilis* could be aided by the presence of other host plants and staggered planting of beans. In the study areas, beans are grown at different times in seasons as one of the cultural practices to manage BLBs [17], and this

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practice has the potential to promote BLB migration between gardens. However, there is no information to confirm whether the presence of beans in the gardens attracts BLBs feeding on beans from far way gardens. It may also be hypothesized that BLBs prefer some bean varieties to others leading them to migrate between gardens. Therefore, it may be important to study whether there are volatiles in bean plants that could attract BLBs leading to their migration. Bean leaf beetles are known to oviposit in the soil and eggs later hatch into larvae, pupae and finally adult [2,17]. However, it remains unclear if these eggs can be moved from one garden to another with the aid of farm implements such as pangas, hoes, gum boots, etc.

Estimates of effective number of migrants per generation (Nm = 13.278) in our *O. mutabilis* samples supported high gene flow that could prevent genetic differentiation [59,22,14]. Our result from IBD analysis also suggests that there are few or no barriers to free movements among *O. mutabilis*. In a study by [27], the furthest an individual *C. trifurcata* beetle flew was 4.9 km. These short flights undertaken while looking for mates, oviposition sites, and food [27] could contribute to broader spatial coverage over time that could lead to low genetic differentiation and high gene flow findings [57]. Information on behavioural studies could help identify factors contributing to the similar findings relating to IBD and gene flow in our target species.

The Rift Valleys in East Africa are known to afford opportunities that could lead to population sub-structure and/or incipient speciation in both invertebrates (e.g., [11,47,28] and vertebrates [13,20]. Whether the geographic spread of *O. mutabilis* that encompassed the Rift Valley could have resulted in similar incipient speciation as identified in the cassava whitefly *Bemisia* 'SSA1' species via a whole genome analysis approach [11] remains to be investigated. Mitochondrial DNA markers such as the partial *COI* gene are not suitable for differentiating between closely related sub-species [6,3,11]. In this study, results from the STRUCTURE analysis detected genetic admixture from two ancestral genetic lineages in our *O. mutabilis* samples, while mt*COI* identified all *O. mutabilis* samples as belonging to a single species. Our findings based on both limited nuclear markers and the maternally inherited mt*COI* marker therefore provided clues that the two ancestral *O. mutabilis* genetic clusters could potentially represent populations undergoing incipient speciation. In-depth population structure analysis based on whole genome sequencing approach for *O. mutabilis* would be needed to provide greater understanding of the evolutionary genetics and landscape adaptation in this significant agricultural coleopteran pest complex.

Difficulty in distinguishing species via morphological characters is an impediment to effective pest management. *Ootheca mutabilis* and *O. proteus* are both serious bean pest species that are morphologically indistinguishable, that can be classified through dissection and examination of the male genitalia [26], a method that fails to differentiate between female species. Morphologically challenging (e.g., [5,39] and cryptic species (e.g., [10] including species within the Coleoptera (e.g., [53] can be readily identified via the mt*COI* gene. Compared to morphological identification, molecular identification using mt*COI* partial gene as outlined in this study successfully distinguished between *O.*

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mutabilis and *O. proteus* that could not be distinguished by colour appearances alone during laboratory sample selection and should be applied to future evolutionary genetic studies of *Ootheca* species.

5. Conclusions

The five microsatellite markers developed in this study are the first such markers for *O. mutabilis*, although their transferability to other *Ootheca* species (e.g., *O. proteus*, *O. orientalis*, *O. bennigseni*) remains to be investigated. This is the first such study to explore the population genetic structure of *O. mutabilis* using mt*CO1* and SSR markers. As demonstrated here, future studies involving *Ootheca* species should first characterize samples via DNA barcoding in addition to phenotypic characterization before population genetic studies to minimise misinformation. The study detected high levels of gene flow among populations of *O. mutabilis* and low but significant genetic differentiation in the sampled agro-ecological zones, with the Ugandan population likely representing a single panmictic population with genetic contributions from two ancestral genetic clusters. Our study provides a baseline for future evolutionary and functional genomic studies to generate a better understanding of host-plant adaptation, insecticide resistance management, and for development of IPM control measures for this pest.

Supplementary Materials: The five novel microsatellites developed (Genbank: MT074093 - MT074097) and the twenty *Ootheca mutabilis* mt*COI* partial gene sequences (Genbank: MW278873 - MW278892) are available in the NCBI.

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