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Research Article

IDENTIFICATION OF HAPLOTYPES OF SITOPHILUS ZEAMAYS POPULATIONS IN THREE AGRO-ECOLOGICAL ZONES

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ABSTRACT

Storage systems are made vulnerable by insects that reduce the value of foodstuffs. In the case of maize, for example, grain losses can be substantial if nothing is done.The aim of this study is to identify the different haplotypes circulating in three agro-ecological zones of Senegal, such as the groundnut basin, eastern Senegal and the middle Casamance, and also to determine the demographic evolution of the populations obtained.To achieve this, samples from all three areas were subjected to mass rearing. Each population was constituted by an average of ten individuals from each sampled locality. DNA extraction, Polymerase Chain Reaction (PCR) and sequencing were carried out to obtain sequences. Parameters such as neutrality indices, mismatches distribution, and the haplotype network were determined using genetic software. The results obtained enabled us to identify 14 haplotypes circulating in these three agro-ecological zones, 10 of which are individual. Haplotype (H1) is the most represented and is common to all three zones. The results show a certain influence of the zones on the demographic structure of the populations studied.

Keywords: Haplotype, demographic parameters, Sitophilus zeamays, genetic diversity, expansion, equilibrium, Senegal, agro-ecological zone.

INTRODUCTION

Genetic diversity is a measure quantifying the extent of genetic variability within a population **[1].** According to Frankham *et al.,***[2]**, it is the raw material on which selection acts to enable adaptation to environmental changes, and hence evolution. Depending on environmental factors, genetic variability is the source of variation in observable characteristics such as shape, size, etc. It thus enables species to adapt to a constantly changing environment and to resist parasites. Genetic diversity is expressed by the frequencies of the different alleles at a locus. According to Ellegren and Galtier **[3],** genetic diversity is generated by the appearance and disappearance of genetic variants called alleles. Within each species, different alleles coexist for a given gene. These alleles have appeared over time, as a result of germline mutations that have spread through populations. It should also be noted that the maintenance of these alleles can be influenced by natural selection, genetic drift and migration. The combination of alleles from two or more polymorphic loci on the same chromosome corresponds to a haplotype. According to Muths **[4],** the term haplotype corresponds to a nucleotide sequence that may be common to several individuals, but differs from other haplotypes by one or more nucleotide substitutions. The study of haplotypes is biologically relevant, since they are a reflection of evolution and correspond to more complex information than individual polymorphisms. However, living conditions can be a determining factor in the diversification of species and individuals.

This is why it would be interesting to highlight the circulation of haplotypes within *S. zeamays* populations in three differentagro-ecological zones. The aim of this study was to identify the different haplotypes encountered in the three agro-ecological zones studied, and to determine the demographic evolution of the different populations studied.

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MATERIALS AND METHODS

Sampling and mass rearing

The samples came from three locations: two in Senegal (KeurAyib and Missirah) and one in Guinea Konakry in the Labé region.

For each locality, around 1 kg of maize(*Zea mays*) was taken from our producer partners, either in the granaries or in the fields. These samples, which sometimes begin to infest on the spot, were brought back to the laboratory and placed in 16 cm high, 9 cm diameter jars with ventilated lids. The samples were then stored for mass rearing. In fact, mass rearing involves allowing the insects to reproduce for at least two generations in order to increase the population sampled. The insect pests of the species *Sitophilus zeamays* were then collected and preserved in 96° alcohol in the laboratory for molecular biology purposes.

The samples were coded by taking the first capital letter of the genus name *Sitophilus (S)*, then the first two letters of the locality (the first capital letter and the letter immediately following it in lower case) and finally capitalizing the first letter designating the colour of the type of maize collected in this locality (Table 1). The individuals from samples from the same locality form a population. This corresponds to a total of five (5) populations. The size of the populations studied depends on the number of individuals taken per sample; this number varies from one locality to another. The population in the groundnut basin is therefore made up of insects from maize plants with different phenotypes.

Table 1: Summary of sampling

Localities	Geographical coordinates		Agro-ecological zones	Agro-climatic zones	Pheno-types of maize	Codes samples	Number of samples
	Latitude	Longitude					
KeurAyib	13° 35'N	15° 36'W	Groundnut basin	Sudanese	MY	SKeY	05
					MM	SKeM	04
Labé	11° 19'N	12° 16'W	Average Guinea	Foutanienne	MY	SLaY	10
Misrah	13° 31'N	13° 30'W	Eastern Senegal	Sub-Guinean	MM	SMiM	12

DNA analysis

Cytochrome B (*Cyt-B*)

The Cyt-B mitochondrial gene is one of the most widely used genes in studies on the molecular evolution and structuring of insect pest species **[5; 6]**. The mtDNA is haploid (N) and non-recombinant **[7]**. It is transmitted exclusively by the maternal organism.The mtDNA reveals more specific variability than nuclear DNA. Consequently, the gene encoding Cyt-B is widely used in molecular phylogeny **[8; 9]** and also in population genetics **[10]**.

Mitochondrial DNA has been used extensively as a tool for deciphering the evolutionary and demographic history of populations and species history of populations and species **[11]**.

Extraction of *S. zeamays* DNA

Extraction involves four steps: tissue digestion, cell lysis, DNA purification and elution.In our study, we extracted DNA from S. zeamays insect tissues using the zymo research kit, following the standard protocol. For each individual, the head, thorax and legs were removed and placed in a 1.5 ml tube.

DNA amplification by polymerase chain reaction (PCR)

For DNA amplification by PCR, the forward and reverse primers used were mtD26 (5'-TATGTACTACCATGAGGACAAATATC-3') and mtD28 (5'-ATTACACCTCCTAATTTATTAGGAAT-3') respectively.

PCR was performed using the One Taq Quick-Load 2X Master Mix kit in a 25-µl reaction volume containing 12.5 µl of Master Mix, 08.5 µl of pure water, 1 µl of primers (i.e. a volume of 0.5 µl per primer) and 1 µl of $MgCl_2$ as catalyst.The amplification conditions were as follows: (i) polymerase activation (hot start) and initial denaturation for 03 minutes at 94°C, (ii) 35 cycles of denaturation at 94°C for 01 minute followed by one minute at 47°C for hybridisation and primer extension or elongation for one minute at 72°C and (iii) final elongation at 72°C for 10 minutes.

Sequencing

DNA sequencing consists of determining the order in which the nucleotides of a given DNA fragment are linked together. In our study, sequencing was carried out by a South Korean company called Macrogen. The gene sequenced was cytochrome B, a mitochondrial gene of great interest.

Genetics analysis

Sequences alignment, cleaning and correction

According to Swofford *et al.,***[12]**, sequence alignment is important in determining whether or not sites are similar. The sequences were aligned as a whole using BioEdit version 7.2.5 **[13]**. Errors within the sequences were identified and corrected manually using the same software. As corrections were made, the alignment was repeated using the crustal w algorithm **[14]**, which is one of the so-called global alignment methods.

Demographic evolution

The demographic history of *S. zeamays* in Senegal was studied by performing demogenetic tests such as Tajima's D, Fu's Fs, Raggedness's irregularity index (rg) as well as Ramos's R2, and by analyzing the distribution of mismatches.

The Tajima's D and Fu's Fs **[15; 16]** were used to test the deviation from the neutrality hypothesis using DNASp 5.10.01 software **[17]**. These indices are known to be sensitive to departures from mutation-drift equilibrium due to changes in population size and selection **[18]**. Fu's Fs test compares the average number of pair wise differences with the number of haplotypes (h) in the population. Tajima's D is based on the difference between the mean number of pair wise differences and the number of variable sites.

Under conditions of constant population size, Tajima's D and Fu's Fs should approach zero. Significantly negative values suggest sudden population expansions and positive values suggest bottlenecks. Significantly negative Fs values and not negative D values suggest recent demographic expansion, while the opposite suggests selection. Significance is obtained by determining their p-value, which is compared with an alpha threshold of 0.05 (p-value > alpha: not significant; p-value < alpha: significant).

The Ramos R2 statistic **[19]**, based on the differences between the number of singleton mutations and the mean of the nucleotide differences, was calculated using DNASp 5.10.01 **[17]**. In a complementary manner, the R2 statistic of Ramos **[19]**, based on the differences between the number of singletons mutations and the average of the nucleotide differences, was calculated with DNASp 5.10.01 **[17]**. Ramos - Onsins and Rozas**[19]** argue that Fu's Fs and R2 are the most powerful tests for detecting population growth. Ramos' R2 is better for small populations and Fs for large populations. The Raggedness irregularity index (rg) was also determined using DNASp 5. 10. 01 software **[17]**.

The haplotype network was constructed using Network software version 10.2.0.0**[20].** This method produces an estimate of the plausibility of the links between the haplotypes in the tree, which must be at least 95% for them to be represented.Mismatch distribution analysis or the distribution of the observed number of differences between pairs of haplotypes **[21]** was estimated using DNASp

5.10.01 **[17]**. Expected values were constructed assuming a constant population size. According to Rogers & Harpending **[21]**, recent rapid population growth is characterised by a unimodal distribution, whereas a multimodal distribution characterises a population in demographic equilibrium.

RESULTS

Haplotypes

Haplotype identification

The 31 Cyt-B gene sequences we studied gave a total of 14 haplotypes, 10 of which are unique (H3, H5, H6, H7, H9, H10, H11, H12, H13 and H14). However, we noted the presence of a majority haplotype (H1) made up of 11 individuals. In this haplotype, all localities were represented but individuals from Misrah are in the majority; only one individual from KeurAyib belongs to this haplotype and three are from Labé. The H1 haplotype is the only heterogeneous one, the others are all homogeneous. The KeurAyib population is the least homogeneous; in fact, we observed the greatest dispersion among individuals from this locality; individuals from this locality are found in six different haplotypes(Table 2).

Table 2: **Number and composition of haplotypes**

Haplotypes	Workforce	Individuals
H1	11	SKeM1, SLaY7, SLaY8, SLaY9, SMiM5, SMiM8, SMiM9, SMiM10, SMiM11, SMiM13, SMiM14
H2	03	SKeY1, SKeY3, SKeY4
H3	01	SKeM2
H4	02	SKeY2, SKeY5
H5	01	SKeM3
H6	01	SKeM4
H7	01	SLaY1
H8	05	SLaY2, SLaY3, SLaY4, SLaY5, SLaY10
H9	01	SLaY6
H10	01	SMiM1
H11	01	SMiM2
H12	01	SMiM3
H13	01	SMiM4
H14	01	SMiM12

Geographical distribution of haplotypes

The H1 haplotype is the only haplotype present in all three zones; it is the haplotype that circulates between these three zones. A total of five haplotypes (H2, H3, H4, H5 and H6) circulate in the groundnut basin zone; these are private haplotypes. Haplotypes H7, H8 and H9 are also only present in the Casamance zone, while H10, H11, H12, H13 and H14 are only present in the Eastern Senegal zone. Haplotypes H1, H2 and H8 are more represented in eastern Senegal, the groundnut basin and Casamance, with representativeness rates of 59 %, 34 % and 50 % respectively (Figure 3).

Demographic expansion

Neutrality indices

Considering the total population, Fu's Fs and Tajima's D are negative and insignificant (*P*-values > 0.10). However, Ramos' R2 and raggedness (rg) are also positive but insignificant (P-values > 0.10), (Table 3).

Table 3: **Demographic parameters of the total S. zeamays population**

	Fu's Fs	Tajima's D	Ramos'R2	rg
Values	-2.44082	-1.08429	0.121325	0.041961
P-values	0.163	0.156	0.599	0.218

With regard to agro-ecological zones, we found that Tajima's Ds, which are not significant, are negative for the zones of eastern Senegal and mid-Casamance, but positive for the groundnut basin. Fu's Fs are all positive and insignificant. Ramos' R2 and raggedness are also positive and insignificant (Table 4).

Table 4: Neutrality indices by zone or locality

Localities / Indices	KeurAyib (Bassinarachidier)	Misrah (Sénégal oriental)	Labé (Casamance)
Tajima's D	1.26914	-1.44497	-1.1165
P-value	0.921	0.085	0.126025
Fu's Fs	0.420911	1.20764	1.2373
P-value	0.555	0.735	0.781762
Raggedness (rg)	0.055556	0.12787	0.102222
P-value	0.16	0.659	0.233
Ramos'R2	0.225658	0.178279	0.219024
P-value	0.907	0.742	0.792008

Mismatch distribution

The total population shows a mismatch curve which is multimodal (Figure 2). The mismatch curves for the populations from KeurAyib, Misrah and Labé are all multimodal (Figure 3). The differences between the expected and observed frequencies (solid and dotted lines respectively) are relatively marked and more so with the KeurAyib population, which belongs to the agro-ecological zone of the Groundnut Basin.

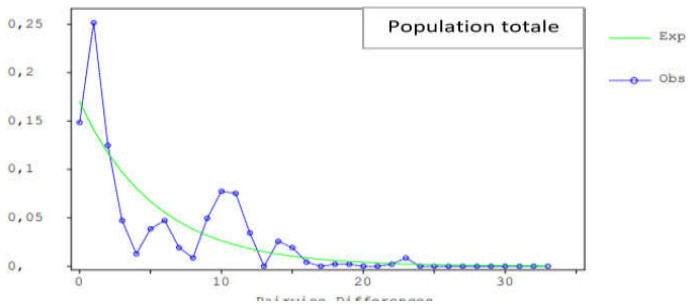

Figure 2: Mismatch curves for the total population showing expected and observed frequencies (solid and dotted lines respectively).

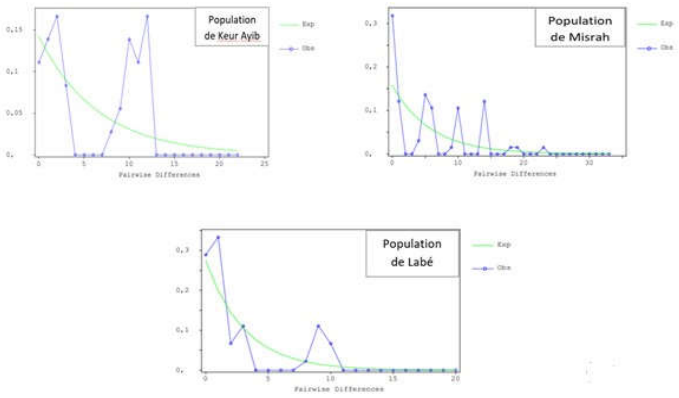

Figure 3: Mismatch distribution curves for the sampled populations showing expected and observed frequencies (solid and dotted lines respectively).

Haplotypic network

The haplotypic network is relatively simple, with a more or less star-shaped pattern starting from the H1 haplotype. This haplotype is in the majority and is central. Five haplotypes branch off from this H1 haplotype, three of which are individual (H12, H5 and H14). All the other remaining haplotypes, individual haplotypes except H4, form a chain starting from H5. We also noted the presence of a mutation step with an undetected haplotype between haplotypes H1, H12 and H14. The appearance of haplotype H14 required more than thirteen (13) mutations (Figure 4).

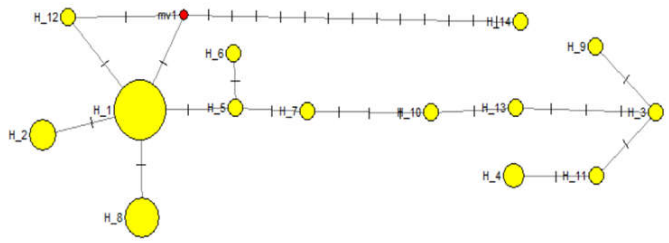

Figure 4: Haplotype network

Each circle corresponds to a haplotype; the size of the circle is proportional to the frequency of the haplotype in the dataset. The length of the segments between each haplotype is proportional to the number of mutations separating them (mutational steps). The red square symbolises a haplotype that was not detected during the study but is necessary for the construction of the network (missing haplotypes or haplotypes lost through evolutionary drift).

DISCUSSION

*Sitophilus zeamays*has been the subject of various studies, few of which have been devoted to the genetic aspect. Yet this insect causes enormous damage to maizestocks. In rural areas with poorly developed food preservation techniques, Sitophilus zeamays can cause post-harvest losses of up to 90 % during 5 months of storage**[22]**.

The aim of this study is to determine the haplotypes circulating within populations of this insect pest in three agro-ecological zones of Senegal using the Cyt-B mitochondrial gene. The demographic evolution of the populations was also determined.

Analysis of the Cytochrome B (*Cyt-B*) mitochondrial gene sequences obtained showed that the *S. zeamays* populations studied contain haplotypes that are different. Fourteen (14) haplotypes of cowpea bruchid circulate in all three agro-ecological zones sampled (Groundnut Basin, Middle Casamance and Eastern Senegal). The level of genetic variability is high, with a total of 14 haplotypes out of the 31 sequences analysed. These results are comparable to those obtained by Kébé **[23]** on the insect *Callosobruchus maculatus*. Mutations therefore induced the appearance of distinct haplotypes. As a result, we have a fairly high number of individual haplotypes; 10 in total out of the 14 obtained in this dataset. This is confirmed by Grant and Bown **[24]**, who, considering the levels of diversity detected through two indices, argue that under conditions of rapid expansion, haplotypic diversity grows more rapidly than nucleotidic diversity, leading to a large number of single haplotypes. Generally speaking, we can see connectivity between the different populations, which means that there has been gene flow between them. These populations have therefore exchanged genes with each other. These exchanges are induced by what we might call one or more mixing factors. These mixing factors are the maize seed trade, bartering for new seeds and the fact that the agro-ecological zones studied form a border. Each of these factors can cause mixing between different strains.

The majority haplotype, H1, shows a fairly high level of diversity because its groups together individuals from all the localities studied. This haplotype circulates within the three agro-ecological zones but very little in the Groundnut Basin zone with only one individual representing it. In these zones, haplotypic diversity is not significant except in the Groundnut Basin zone where it is quite high. In this area of the groundnut basin, the large number of haplotypes may be due to the fact that we used maize with different phenotypes (yellow maize and a mixture of yellow and white maize) and also to the fact that the locality of KeurAyib, which represents this area, is a major trading market for foodstuffs such as maize. This genetic diversity could reflect a past bottleneck, followed by rapid population expansion and an accumulation of mutations. These results indicate a signal of a stable total population with a large effective size or admixture from populations that have been isolated from each other **[25]**. This is also in line with the findings of Dia*et al.,***[26]** on other populations of beetles attached to stored or marketed foodstuffs.

In the Casamance area, the H1haplotype circulates on a permanent basis, indicating mixing within the population studied. This population, like that of eastern Senegal, is relatively homogeneous, with a low number of circulating haplotypes. The H1 haplotype is the most widely shared; it was found in all three agro-ecological zones studied. This geographical distribution of the H1 haplotype, given that the three agro-ecological zones studied are border areas, may be due to the marketing of maize seed or the sharing of seed between farmers. As maize is a staple food, it is widely shared between populations during lean periods. This could lead to an exchange or transfer of *Sitophilus zeamays* strains from one stock to another, causing them to mix. This haplotype is also the central one from which many others originate, but it is made up of individuals from all three zones. This result can be explained by the fact that there has been a dispersal of a strain or a mixture of different strains that have had to exchange alleles. In fact, trade is a major factor in the circulation of foodstuffs, especially maize, and so it may allow mixing between strains or even dispersion of a given strain. The geographical distribution of haplotypes seems to show the influence of agro-ecological zones on the diversity or diversification of *Sitophilus* populations. Although we have here a central circulating haplotype, a certain grouping of haplotypes according to their geographical origin is also clearly noted. The demo-genetic parameters reveal that the total population of *S. zeamays* has undergone a recent demographic expansion following a bottleneck. Tajiama's D and Fu's Fs are negative and insignificant. Ramos' R2 and raggedness (rg) are positive and insignificant. However, the mismach multimodal distribution curve indicates a population in equilibrium.

The haplotypic network revealed the presence of numerous unique haplotypic variants present in all the localities sampled (and therefore in the agro-ecological zones studied), as well as the presence of common haplotypes of which H1 is the central one. The distribution found does not show an obvious grouping of individuals in relation to their sampling locality, but rather a grouping according to genetic origin materialised by the H1 haplotype. In other words, the structure of the network appears star-shaped, with numerous haplotypes that are infrequent and not very divergent, and a fairly large number of individual haplotypes. Generally speaking, we can say that there has been connectivity between the different populations. This means that there is a flow of genes between them. But we can also see, through the individual haplotypes, a high level of intra-population diversity, which may also be due to the effect of maize marketing. Marketing

tends to mix different strains, hence the diversity observed between populations. Over time, this mixing of strains will lead to regular cross-breeding and genetic homogenisation, which could result in a common majority haplotype following further demographic expansion. Subsequently, these populations will end up being homogeneous as cross-breeding proceeds. The presence of a central haplotype (H1) provides information about the link between the different populations. In fact, we can say that it is from an original population that the others derive, and they begin to stand out according to their area of existence. The agro-ecological zones considered would have an impact on influence on the structure of the *S. zeamays* populations that develop there. This observed generalist structuring could be explained by the fact that *S. zeamays* behaves like a panmictic unit. This aspect of the structure is consistent if we consider the populations studied separately. The KeurAyib population has positive but no significant Tajima's D and Fu's Fs values. These Tajima's D and Fu's Fs values reflect populations that are in equilibrium. For the Misrah and Labé populations, which have a positive Fu's Fs and a negative Tajima's D, but all non-significant, this indicates selection. These results give a total population that does not have a well-defined structure. However, this structure is not even confirmed by the other demographic parameters calculated, such as the Raggedness irregularity index (rg) and Ramos' R2. The positive and not significant rg for all the populations considered reflects a recent demographic expansion. The positive and not-significant Ramos' R2 also indicates a rather moderate demographic expansion.

On the other hand, the mismatch curves, which are multimodal, reveal that all the populations studied are in equilibrium.

CONCLUSION

Subject to a more in-depth study, with a larger number of samples, a number of ideas can be retained. The results show 14 haplotypes out of the 31 sequences analyzed. Of these haplotypes, 10 are individual. This corresponds to an average circulation of haplotypes within the populations studied. The demographic parameters reveal populations in equilibrium. Similarly, the positive values of these tests may indicate not only selection equilibrium but also the presence of a strong population structure, which may increase the proportion of intermediate frequency alleles in these populations. All these populations show an ancestral link with a common, majority central haplotype. This shows the influence of environmental conditions on the biology of *S. zeamays*. Could these results reflect well-defined diversity, genetic structuring and phylogenetic relationships? Wouldn't knowledge of the demographics of this insect make it possible to develop more effective control methods that are more appropriate to nature?

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