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Genetic study of two populations of Sitophilus oryzae following two types of maize: Yellow maize and yellow-white maize from two different localities in Senegal

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ABSTRACT

The objective of this study is to determine the genetic diversity and structure, demographic evolution and phylogenetic relationships of *Sitophilus oryzae (S. oryzae)* in two types of maize: yellow maize and yellow-white maize from two localities in Senegal. Thus, individuals of *S. oryzae* from each locality are used, after sampling and massive rearing. The Cyt-B gene of the mitochondrial DNA (deoxyribonucleic acid) was targeted and sequenced. The obtained sequences are corrected, aligned and exploited. Overall, haplotypic diversity (Hd) is non-significantly high (0.895 ±0.058) while nucleotide diversity (Nd) is significantly low (0.016±0.003), even when populations are considered separately; the Salemata population (Hd=0.949 ±0.051, Nd= 0.015±0.003) is more diverse with P-values≤0.05. Results confirmed by polymorphism with a high number of singletons and haplotypes. This genetic variability can be explained by the fact that we had two different substrates but phenotypically very similar. The parameters of genetic differentiation, demographic and phylogenetic evolution revealed a weak genetic structuring of *S. oryzae*, populations in demographic expansion and poorly defined parental relationships between populations. This study provided further insight into the genetic characteristics of *Sitophilus*.

Contribution/ Originality: This article contributes to the search for solutions for the control of insect pests of foodstuffs. The determination of the genetic parameters of *S. orizae* following two different substrates is interesting. Indeed, the substrate could influence the behavior of the insect. This shows the originality of this article, confirmed by our results.

1. INTRODUCTION

In the Sahel, cereals and legumes are the staple food of the population despite current yields that are far below expectations for national food coverage [1]. The lack of food resources has always been met by massive imports, particularly of cereals. According to the Programme Alimentaire Mondial (PAM) [2] cereal products represent 56% of food imports, or 1.5 million tons. Despite initiatives on both sides to increase production, the context of food insecurity is still marked by significant post-harvest losses. In Canada, for example, a list of insect-tolerant corn and the pests they target is available and is maintained by the Canadian Corn Pest Coalition [3]. Indeed, between harvest and consumption, more than 30% of the production is lost, this proportion being higher in the Sahelian region due to the long storage period [4]. Maize *(Zea mays, L.)* is a staple food for many African countries [1].

Maize is widely cultivated as a cereal for its starch-rich grains (72 to 73% of its weight) and represents the first cereal production before rice and wheat [5, 6]. In recent years, millet and maize have become very present in the agri-food sector in Senegal [7]. Maize *(Zea mays L)*, the world's leading cereal ahead of rice and wheat, is however the second major cereal after millet in Senegal. However, the efforts made to progressively remove constraints and improve production may prove useless without an adequate post-harvest conservation system [8]. This makes the protection of stocks a thorny issue in many respects. Like all cereals, corn is not spared by insect pests of stocks more particularly by the genus *Sitophilus*. Commonly called grain weevil, the species *Sitophilus oryzae (S. oryzae)* is considered together with *Sitophilus zeamais (S. zeamais)* as the most damaging insects of stocks in tropical countries. *S. zeamais* prefers large grains such as maize while *S. oryzae* has a preference for small grains such as rice [9]. However, the effect of *S. oryzae* on maize should not be overlooked. In view of the serious threat posed by these insect pests to maize stocks, a sound knowledge of their biology is of utmost importance; this could lead to the development of more effective control strategies. Thus, by analyzing mitochondrial DNA (desoxyribonucleic acid) extracts, specifically the cytochrome B (Cyt-B) gene from individuals of *S. oryzae*, we will determine the genetic diversity and structure of this species in two types of maize: yellow maize and yellow-white maize on the one hand, and on the other hand, we will also determine their demographic evolution and phylogenetic relationships.

2. MATERIAL AND METHOD

2.1. Sampling and Mass Rearing of S. Oryzae

Samples were taken from two localities in Senegal: Salemata, located in the Kedougou region and belonging to the agro-ecological zone of eastern Senegal, and Diaroume, located in the Sedhiou region and belonging to the agro-ecological zone of Casamance. For each locality, approximately 1 kg of maize is collected from our producer partners, either in the granaries or in the fields. These samples, which sometimes start to become infested on the spot, are brought back to the laboratory and put in jars (16 cm high and 9 cm in diameter) with a vented lid. They are kept for mass rearing.

It should be noted that these two agro-ecological zones are adjacent to each other, even though the locations chosen are somewhat distant from each other. Mass rearing consists of letting the insects reproduce for at least two generations in order to increase the sampled population. Then the insect pests, of the species *S. oryzae*, are collected and preserved in 96° alcohol in the laboratory for molecular biology purposes.

The coding of the samples was done 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 capitalize the first letter designating the color of the type of maize collected in this locality. Example: SSaJ stands for *Sitophilus (S)* Salemata (Sa) yellow maize (J), (see Table 1).

Table 1. Summary of the sampling.

Areas	Locations sampled	Type of corn	Sample codes	Number of individuals	Geographical coordinates of localities	
					Latitude	Longitude
Kedougou	Salemata	Yellow corn(J)	SSaJ	13	12°37'51"N	12°49'03"W
Sedhiou	Diaroume	Yellow-white corn (M)	SDiM	11	13°03'31"N	15°38'20"W
Total				24		

The individuals coming from the samples of the same locality form a population. This corresponds to a total of 2 populations. The size of the populations studied is a function of the number of individuals taken by sample which is also a function of the means available; this number is variable according to the localities.

3. DNA (DEOXYRIBONUCLEIC ACID) ANALYSIS

3.1. Cytochrome B (Cyt-B)

One of the most regularly used genes in studies on the molecular evolution and structuring of insect pest species is the mitochondrial Cyt-B gene [10, 11]. It is a mitochondrial gene. The mitochondrial genome or mtDNA (mitogenome), is made up of a circular DNA molecule of simple structure and contained in the mitochondria which are like clones due to their bacterial type reproduction. The mtDNA is haploid (N), not recombinant [12]. It is transmitted almost exclusively through the maternal body. The mtDNA is more suitable for demonstrating specific variability than nuclear DNA. The gene encoding Cyt-B is widely used in molecular phylogeny [13, 14] and also in population genetics [15]. Despite the fact that Cyt-B is subject to strong evolutionary constraints, some of its internal regions are more or less conserved than others due to their functional restrictions [16]. In molecular biology, the most used mtDNA regions are the coding sequence of the cytochrome B gene.

3.2. Extraction of DNA from Sitophilus Orizae

Extraction is done in four steps: tissue digestion, cell lysis, DNA purification and elution. In our study, we extracted DNA from tissues of the insect Sitophilus orizae with the zymo research kit following the standard protocol. For each individual, the head, thorax and legs were collected and placed in a 1.5 ml tube.

3.3. DNA Amplification by Polymerase Chain Reaction (PCR)

In the PCR amplification of DNA, the forword and reverse primers used were mtD26 (5'-TATGTACTACCATGAGGACAAATATC-3') and mtD28 (5'-ATTACACCTCCTAATTTATTAGGAAT-3'), respectively. PCR was performed with 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, 1µl of the primers (i.e., a volume of 0.5 µl per primer), and 1 µl of MgCl2 as catalyst. The amplification conditions are: (i) a polymerase activation (hot start) and initial denaturation step of 03 minutes at 94°C, (ii) 35 cycles of denaturation at 94°C for 01 minute followed by a one minute time at 47°C for hybridization and primer extension or elongation for a one minute time at 72°C and (iii) comes a final elongation at 72°C for 10 minutes. The amplification conditions are shown in Appendix 1.

3.4. Séquencing

DNA sequencing consists of determining the sequence of nucleotides in a given DNA fragment.

In our study, the sequencing was performed by a South Korean company called Macrogen. The gene sequenced is cytochrome B, which is a mitochondrial gene of great interest. Macrogen's analysis protocols are fully standardized and partly automated, which allows a homogeneity of results and a better yield. For this purpose, we have the tubes containing the PCR products and the primers at the said company.

4. GENETIC ANALYSIS

4.1. Sequence Alignment, Cleaning and Correction

According to Nei [17] sequence alignment is important in determining whether sites are similar or not. After sequencing, the resulting sequences were aligned and corrected. This starts with a check of the correspondence between the chromatogram and the sequences, which is sometimes not well established in some parts. Then the sequences were aligned as a whole using the Bio Edit ver. 7.2.5 software [18]. This operation allowed to prune the beginnings and ends of the sequences which were often very improper due to the hooking and detaching of the primers. Errors within the sequences were identified and corrected manually using the same software. As the correction was made, the alignment was renewed using the crustal w algorithm [19] which is part of the so-called global alignment methods.

4.2. Analysis of Genetic Diversity

Haplotypic (gene) diversity is defined as the probability that two randomly selected alleles or haplotypes in a sample are different [17], while nucleotide diversity is the probability that two randomly selected homologous nucleotide sites are different. These parameters were used to determine the genetic variation of *Sitophilus oryzae*.

Since the gene under study is Cyt-B; a mitochondrial gene, we first checked the codon structure of the gene sequences by transforming them into amino acids using MEGA7.0.14 software [10]. This operation revealed no evidence of putative nuclear pseudogenes. Then the parameters like the number of polymorphic sites, the number of informative sites in parsimony, the number of singletons, the number of haplotypes as well as the haplotypic and nucleotide diversities were determined using the DNASp software version 5.10.01 [20].

4.3. Genetic Structuring

The genetic structuring of *Sitophilus oryzae* was determined from the following genetic differentiation parameters: these are genetic distance and Fst. The genetic distance between localities was calculated by the software Mega 7 version 7.0.14 [10] using the Kimura 2-parameter model (K2P) [21]. DNA sp software version 5.10.01 [20] was used to determine the Fst values. A permutation test (1000 boostraps) was applied following the approach described by Excoffier and Heckel [18] to assess the significance level of locality pairwise differentiation.

4.4. Demographic Evolution

The demographic history of the populations sampled in the different localities was apprehended from a "mismatch distribution" analysis of the populations. This analysis is supported by the evaluation of demographic tests such as Tajima's D, Fu's Fs of Ramos' R2 and raggedness (r). The values of D de tajima, Fs de Fu were calculated by the software Arlequin 3.5.13 [18]. While those of Ramos' R2 and raggedness were calculated by DNA sp software version 5.10.01 [20].

4.5. Phylogenetic Relationships

Phylogenetic reconstruction allows us to clarify the existing relationships between haplotypes identified in the different populations. Thus, in our study, we constructed two phylogenetic trees, one according to the neighbor joining (NJ) method and the other by maximum likelihood (ML), using the software Mega version 7.0.14 [10]. The comparison of these 2 trees allowed to verify the coherence of the interpretation of the phylogeny of the populations. Reconstructions were rooted with a homologous sequence of the species *S. zeamais*. These trees are reinforced by the development of a haplotype network. This will allow to better see the phylogenetic relationships that can exist between these populations. With the software of Network version 10.2.0.0 [22] the network for haplotypes of *S. oryzae* sequences was constructed.

5. RESULTS

5.1. Genetic Diversity

5.1.1. Polymorphism

Analysis of the constituent sequences of the two *Sitophilus oryzae* populations studied showed the following results:

Overall, the 24 sequences of the global population are each composed of 410 bp. corresponding to the total number of sites. Among these sites, 386 conserved sites, 24 variable sites of which 19 sites are informative in parsimony and 5 are singletons sites. If we take the two populations separately, we find that the Diaroume population presented 391 conserved sites out of the 410 studied, 19 variable sites of which 15 are informative and 4 are singleton sites. For the Salemata population, 388 sites are preserved and 22 are variable. Among these variable sites, we noted many singletons; 12 singletons sites in total, whereas the polymorphic sites are 10.

The number of singleton sites is very high for the Salemata population (12 sites) while they are rather low for the Diaroume population (only 4 sites). The total population also has a low number of singleton sites (05 sites). Table 2 shows the results of the genetic polymorphism parameters of the study populations.

Table 2. Summary of genetic polymorphism parameters.

Populations / Parameters	Diaroume	Salemata	Overall population
Number of sequences	11	13	24
Total number of sites	410	410	410
Preserved sites	391	388	386
Variable sites	19	22	24
Singletons sites	04	12	05
Informative sites	15	10	19

5.1.2. Genetic Variability

The sequences studied showed a relatively high number of haplotypes for the two populations taken separately, whereas for the overall population, the number of haplotypes is very high (16 out of 24 sequences in total), with a single majority haplotype composed of 8 individuals (5 from Diaroume and 3 from Salemata). Table 3 shows the number of haplotypes encountered and the number of individuals constituting each of them.

Table 3. Number and composition of haplotypes.

Haplotypes	Workforce	Individuals
H1	01	SDiM1
H2	01	SDiM2
H3	01	SDiM3
H4	01	SDiM4
H5	01	SDiM5
H6	01	SDiM6
H7	08	SDiM7, SDiM8, SDiM9, SDiM10 SDiM11, SSaJ3, SSaJ5 et SSaJ10
H8	01	SSaJ1
H9	01	SSaJ2
H10	01	SSaJ4
H11	01	SSaJ6
H12	02	SSaJ7 et SSaJ13
H13	01	SSaJ8
H14	01	SSaJ9
H15	01	SSaJ11
H16	01	SSaJ12

Note: Code: Example: SSaJ stands for *Sitophilus (S)* Salemata (Sa) yellow maize (J).

The overall haplotypic diversity is high (0.895) and is barely significant (P = 0.058) while its nucleotide diversity is significantly low (0.01633±0.00255). For the individual populations, namely the Diaroume and Salemata populations, we noted haplotypic diversities that are strong but not significant. However, the P-value for Salemata is very close to significance (P-value = 0.051). On the other hand, their nucleotide diversities are all low and significant (0.01836 ± 0.00356* for Diaroumé, 0.01451 ± 0.00309* for Salemata). The number of haplotypes is very high for these populations studied (see Table 4).

Table 4. Values of genetic diversity parameters.

Populations / Parameters	Diaroume	Salemata	Overall population
Number of individuals	11	13	24
Number of haplotypes	07	10	16
Haplotypic diversity	0.818	0.949	0.895
P- value	0.119	0.051	0.058
Nucleotide diversity	0.018	0.015	0.016
P-value	0.004*	0.003*	0.003*

Note: The sign "*" indicates the value is significant.

6. GENETIC STRUCTURE OF POPULATIONS

6.1. Genetic Differentiation

The values of genetic differentiation (Fst) are calculated considering different levels (Total population, between localities).

Overall the value of genetic differentiation (Fst) of the populations studied is 0.00667 with a P-value of 0.00000±0.00000; thus a significant Fst.

The genetic differentiations obtained between these two populations show Fst values of 0.28829 which is a little high and significant (P-values=0.0402). This shows that these two populations are relatively little divergent.

6.2. Genetic Distance (D)

Table 5 and 6 shows the inter- and intra-population genetic distances of *Sitophilus oryzae* (bottom) and standard errors (top). The genetic distances within populations are 0.019 for the Diaroumé population and 0.015 for the Salémata population. The genetic distance is relatively small, indicating little genetic divergence between individuals from each locality. It should be noted that individuals from Diaroume are more genetically divergent than those from Salemata. The same observation, i.e. a low genetic divergence, was made when comparing the two populations (D = 0.017). The distance values obtained are all significant.

Table 5. Intra-population genetic distances (D) (bottom) and standard errors (top) of Sitophilus oryzae.

Populations	Genetic distance	Standard errors
Diaroume	0.019	0.004
Salemata	0.015	0.003

Table 6. Genetic distances (D) between populations of Sitophilus oryzae (bottom) and standard errors (top).

Populations	Diaroume	Salemata
Diaroume	….	0.004
Salemata	0.017	….

7. DEMOGRAPHIC TRENDS

7.1. Demographic Tests

The Tajima Ds obtained are all positive and non-significant (P>0.05) for all the populations studied with relatively high values for Diaroume and Salemata. Fu's Fs are all non-significant and negative for the total population and for Salemata, but positive for the Diaroume population. The values of Ramos' R2 and Raggedness (r) are positive. The values of these parameters are recorded in Table 7.

Table 7. Demographic parameters of the populations considered.

Populations	Demographic parameters			
	Tajima's D	Fu's Fs	Ramos'R2	Raggedness(r)
Overall population	0.001	- 4.270	0.137	0.042
Diaroume	0.464	0.661	0.185	0.150
Salemata	-0.693	- 2.403	0.077	0.123

Note: This table does not present any significant value, the p-values are all greater than 0.10.

7.2. Mismatch Distribution Curves

The Mismatch distribution curves are all multimodal for all populations considered: total population, Diaroume population and Salemata population (see Figures 1, 2 and 3).

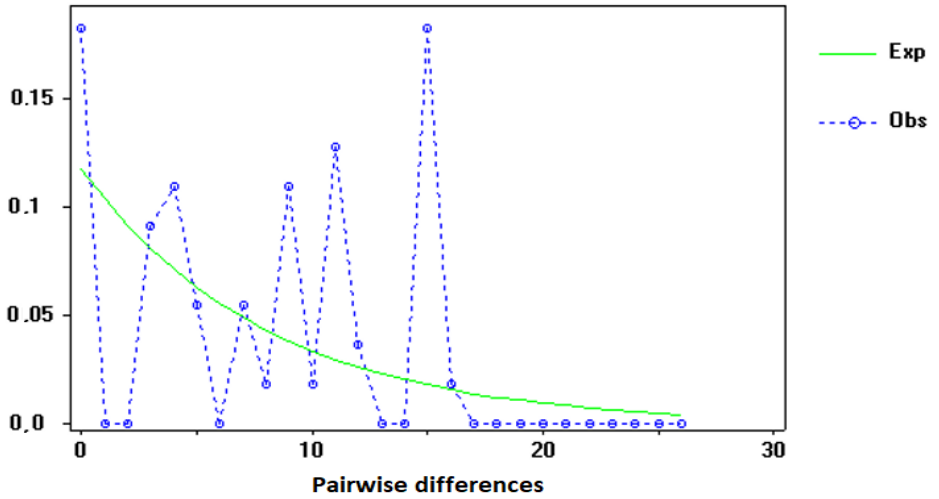

Figure 1. Mismatch distribution curves of the population of Diaroume.

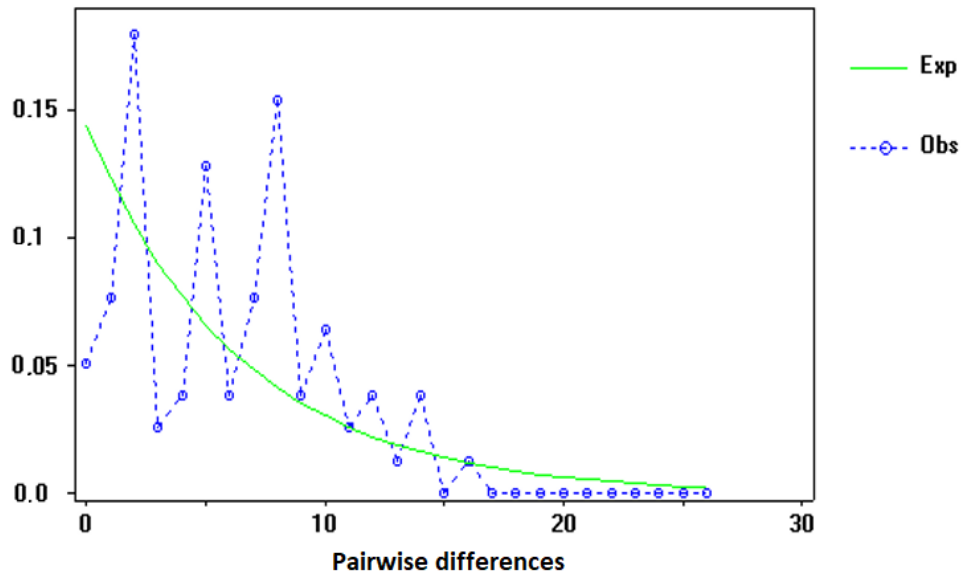

Figure 2. Mismatch distribution curves of the population of Salemata.

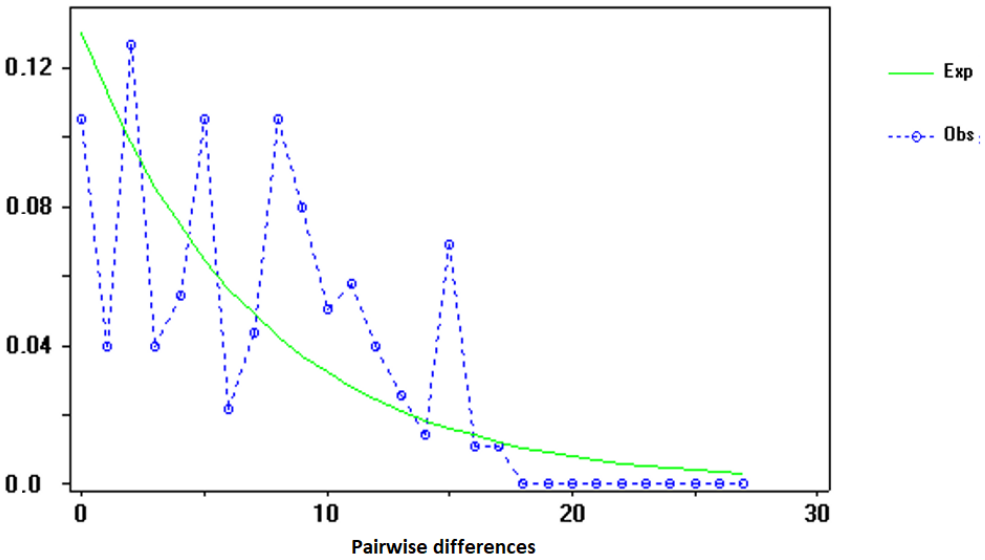

Figure 3. Mismatch distribution curves of the total population.

8. PHYLOGENETIC RELATIONSHIPS

8.1. Phylogenetic Trees

The phylogenetic relationship of these two populations has been appreciated from phylogenetic trees. Here we have considered two methods: the Neighbor joining method and the maximum likelihood method (see Figure 4 and 5).

The two trees are almost similar in terms of the number of groups or clades and the composition of the groups, except that groups 5 and 6 of the Neighbor joining form the fifth group of the maximum likelihood. Group 4 is the only homogeneous group, it is entirely formed by individuals from Salémata. The formation of clades or their distinction into groups was not perfect. The nodes formed are not roobust, with low boostrap values. However, we noted the presence of some isolated individuals such as SSaJ1, SSaJ4, SSaJ6 and SDiM1. These individuals come mainly from the Salemata locality. The phylogenetic relationship is therefore not strong between these two populations of *S. oryzae*.

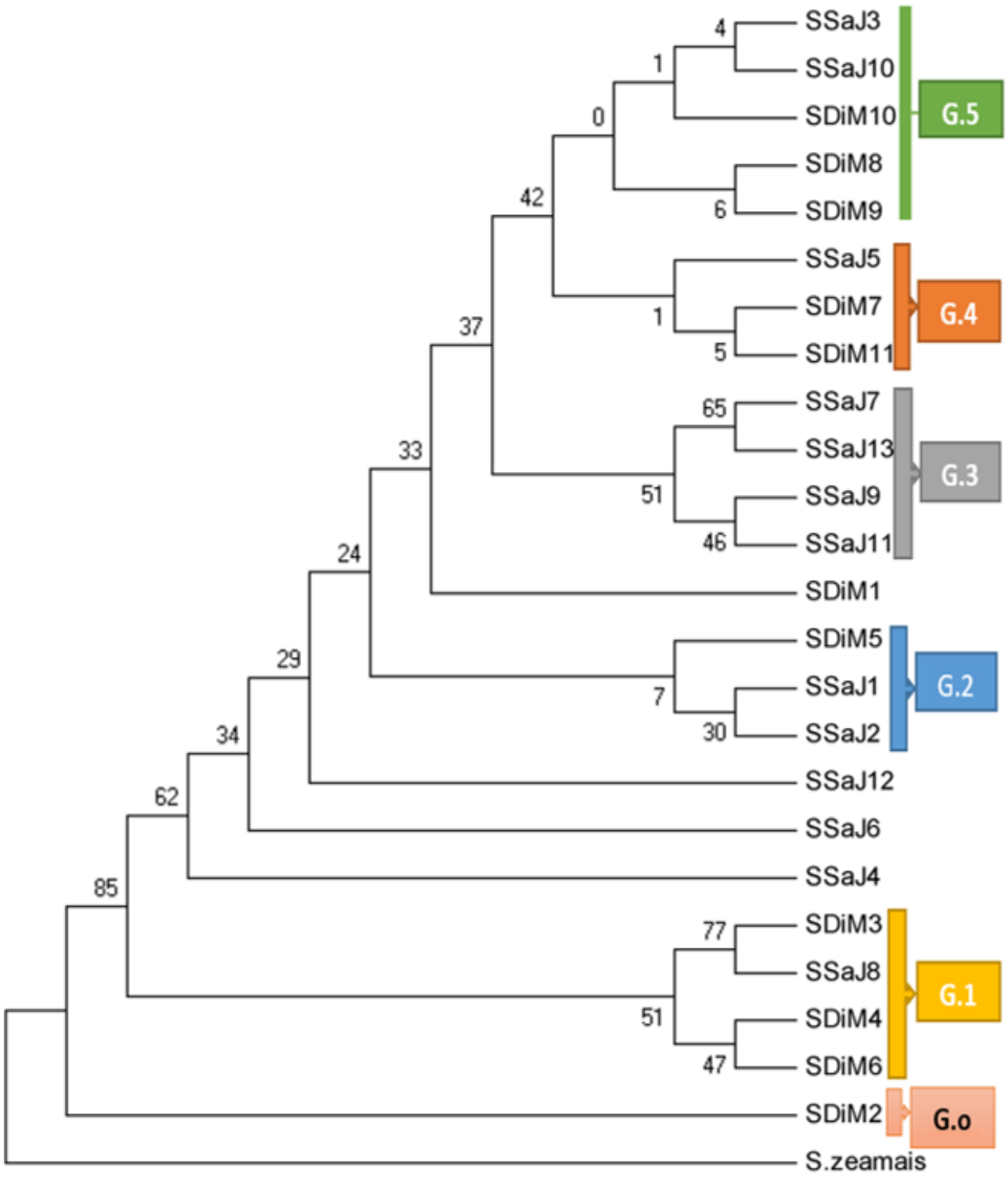

Figure 4. Neighbor joining tree.

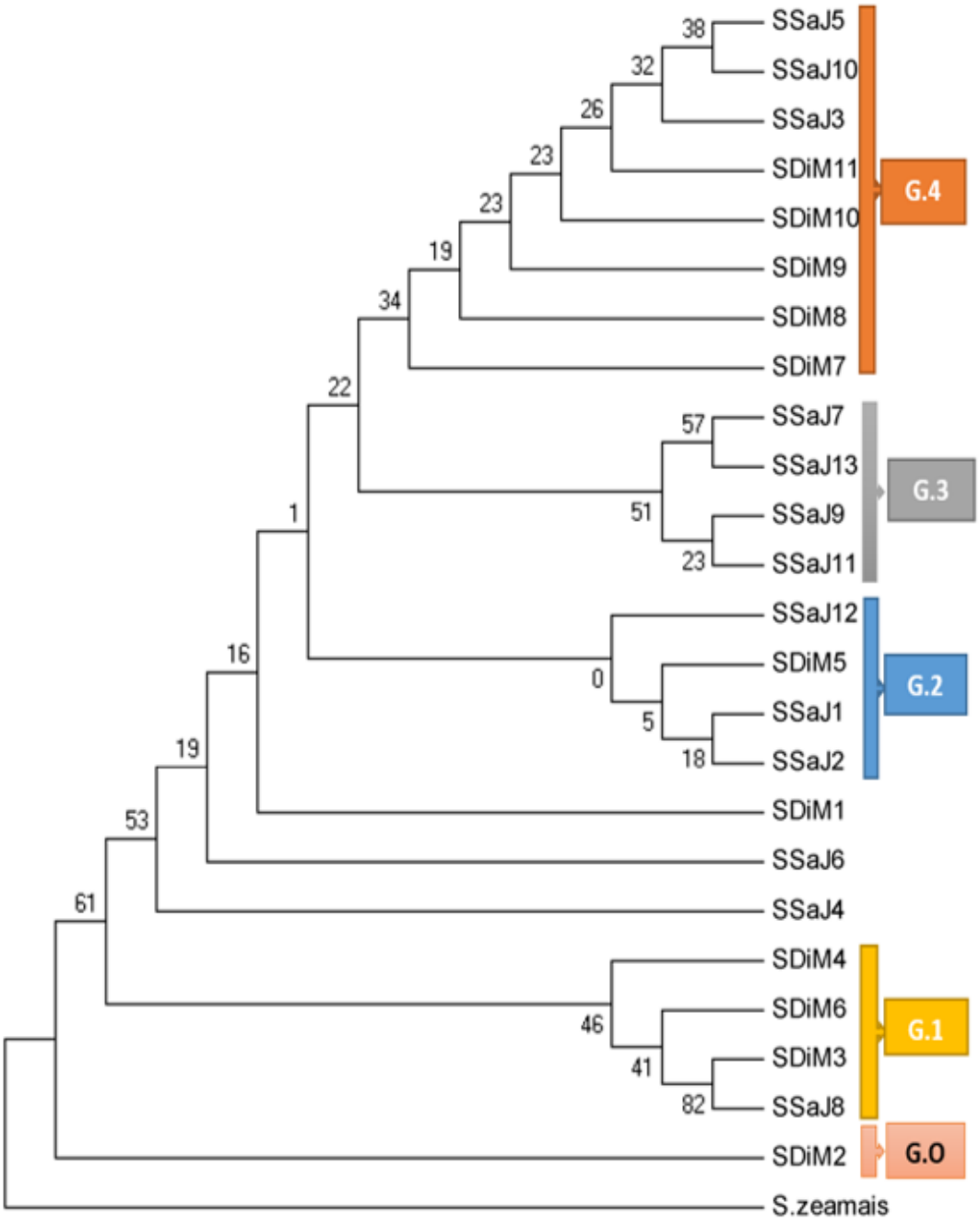

Figure 5. Maximum likelihood tree.

8.2. Haplotype Network

The haplotype network Figure 6, highlights the degree of relatedness between the different samples.

According to the network obtained, the majority haplotype (here the H7 haplotype) is represented by the individual SDiM7 and is made up of individuals from the two localities: Diaroume (majority) and Salemata. To this haplotype are linked other haplotypes that are unique (SDiM1, SSaJ11, SSaJ2) and SSaJ7 which is formed by two individuals and following a mutation step. The H7 haplotype, which is in the majority, is central or ancestral. The network has shown however that the haplotypes existing in these localities are related and present many mutation steps between individuals. Indeed, we have individuals from Diaroume who are directly related to individuals from Salemata and as a result of many mutations. For example, following mutations from the individual SDiM5 from the locality of Diaroumé, we obtained the individual SSaJ8 from Salémata.

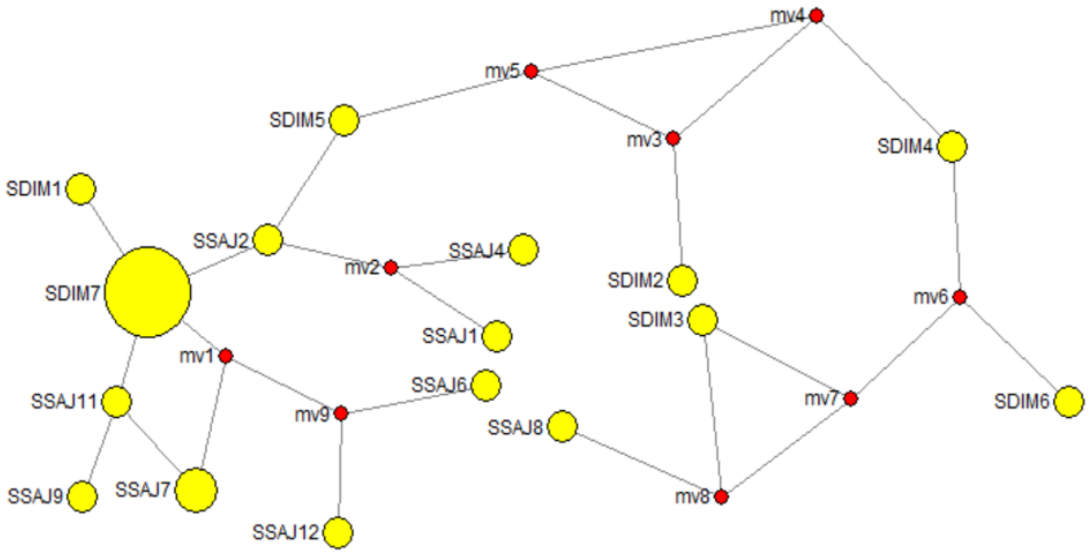

Figure 6. Haplotype network of the two populations.

9. DISCUSSION

The objective of this work was to determine the genetic diversity of *Sitophilus oryzae* following two maize types of different phenotypes using the Cyt-B mitochondrial gene but also to determine their demographic evolution and their phylogenetic relationship.

Indeed, high non-significant haplotypic diversities were observed in all the populations studied while their nucleotide diversities are significantly low.

This high diversity of populations could correspond to a rapid multiplication of the populations studied. Indeed, according to Sinama [23] the high haplotypic diversity and the low nucleotide diversity noted (on mtDNA) may be the result of a rapid growth of populations from an ancestral population with a low number of individuals and for which there has not been enough time elapsed to recover a high diversity between haplotypes. This is confirmed by our results. However, a detailed reading of the results shows that the Salemata population is very diverse from the outset, with P-values ≤ 0.05; thus significant compared to the Diaroume population, which has P-values that are only significant for nucleotide diversity. The polymorphism of these two populations confirms these results. Indeed, the populations studied showed a significant number of polymorphic sites. This finding can be explained by the effect of the substrate which is the type of maize here. Indeed, we can say that yellow maize favors more the diversification of the species *Sitophilus oryzae* than yellow-white maize, since the insects of Salemata, more diversified, are obtained from yellow maize. This is because this type of maize circulates more and is more widespread nationwide. These results reflect a level of genetic and nucleotide diversity that is within the standard ranges of genetic diversity of insect pests [24]. The somewhat similar degree of diversification observed may be explained by the fact that the types of maize used are not too phenotypically distinct. The phenotypic aspect may influence the choice or preference of oviposition substrate. And as we said before, the substrate is a factor of diversification of the species.

Regarding the structure of these populations, the parameters Fst and genetic distance have shown that they are weakly differentiated genetically. This weak structuring is visible both between individuals of a population and between individuals of different populations. There was no clear organizational distinction between these two populations. There is therefore a strong gene flow that has a generally homogenizing effect. This may be due to the effect of marketing, which tends to homogenize the populations of food-stock raptors, since the two localities are geographically distant enough to cause an isolation effect through distance. It should also be noted that these areas are maize producers, which could increase the exchange of seeds in search of seeds and even for food. However, it should be noted that the survival of the insect is not exclusively dependent on the environment because the

endocytobiota (intracellular symbionts) participate in the adaptation of the host *(Sitophilus spp.)* to the variations of the environment [25] which could cause a certain homogeneity of the populations.

Mismatch curves that are multimodal reflect populations that are in equilibrium. The values of Ramos' R2 and the raggedness (r) being positive and non-significant reject the hypothesis of a demographic expansion. This is confirmed for the total population with a relatively null and insignificant Tajima's D (0.00052). The configuration of the haplotype network slightly supports this equilibrium of the total population, but the Tajima Ds of the Salemata and Diaroume populations taken individually reveal a demographic expansion for Salémata (-0.69292) and a bottleneck for Diaroume (0.46374). The Fu Fs of the total population and that of Salemata being negative suggest a demographic expansion event. On the other hand, for Diaroume, the positive Fs of Fu suggests the effect of a positive selection following a recent bottleneck in the population.

From a phylogenetic point of view, the two populations do not show very strong relationships. Therefore, well formed phylogenetic groups do not appear. This idea emerges from the fine reading of the trees constructed, namely the Neighbor Joining tree and the maximum likelihood tree. These trees showed phylogenetic groups not important in size (five individuals on average per group) but also relatively low boostrap values. However, the haplotypic network shows a drift of the populations which can be done from one population to another and in both directions. Indeed the numerous mutation steps observed testify to this drift. The existence of the important number of mutation steps could be the work of environmental factors. This could also be due to the nature of the bridge substrate since the network has shown that a good number of individuals of Salemata are obtained following a mutation step.

10. CONCLUSION

It results from these results that the substrate is an important element which can influence the diversity of the populations. This explains the diversification of these populations but at a different speed according to the type of substrate used, i.e. yellow and yellow-white maize. The relationship between haplotypic diversity and nucleotide diversity informs on the demographic history of the populations. Indeed, we have here populations that in general are in demographic expansion even if certain parameters confirm the idea of a bottleneck for Diaroume. From an organizational point of view, these populations do not present a well-defined genetic structuring or solid phylogenetic relationships. They appear to be generally homogeneous. These assessments obtained on the insect *S. oryzae* may allow better monitoring of populations in stocks. However, studies involving a larger number of populations are needed to reach clearer conclusions.

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Authors' Contributions: All authors contributed equally to the conception and design of the study. All authors have read and agreed to the published version of the manuscript.

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APPENDIX

Appendix 1. Primers used, volume of different reagents and PCR conditions for the Cyt-B gene.

Gene amplified	**Cyt-B**
Primers	mtD26 (5'-TATGTACTACCATGAGGACAAATATC-3')
	mtD28 (5'-ATTACACCTCCTAATTTATTAGGAAT-3')
Reagents	Volume (µl)
Pure water	08, 5
Tampon master mix	12, 5
CB1 (10 µM)	00, 5
CB2 (10 µM)	00, 5
MgCl2	01
Matrix DNA	02
PCR conditions	
Initial denaturation	94°c → 03 min
Number of cycles	35, puis
Denaturation	94°c → 01 min
Hybridization	47°c → 01 min
Elongation	72°c → 01 min
Final elongation	02° c→ 10 min