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Identification of haplotypes of callosobruchus maculatus bruchus populations in different agro-ecological zones of Senegal

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Abstract
The objective of this work is to identify the different haplotypes circulating in the agro-ecological zones of Senegal and to study the demographic evolution of the populations studied.
To do this, we have samples from each zone to be studied. These samples are subjected to massive breeding. Then, on average, ten individuals from each sampled locality were sequenced. These individuals constitute the study populations.
Parameters such as neutrality indices, mismatch and haplotype network are determined using genetic software.
The results showed the presence of 20 haplotypes out of 64 sequences studied. Ten of them are individual and three are majorities (H19, H1 and H4). This shows two homogeneous populations: that of Koki, which constitutes the majority of the H4 haplotype, and that of Tamba, which forms the central H19 haplotype.
According to agro-ecological zones, the Casamance zone has the highest number of haplotypes (08) while the eastern Senegal zone has the lowest number (03).
The demographic parameters show a gene flow between the different populations. Some populations are expanding, such as those of Fouta, Tamba and Koki, which have a negative and insignificant Tajima D and Fu FS, confirmed by SSD, rg and mismatch curves. On the other hand, the other populations are in equilibrium, such as those of Barkédji, Diannah malary, Carrefour diaroumé and Fatick; showing positive Tajima's D and Fu's FS and Fu's FS that are negative only for Diannah malary and Fatick.
Following the agro-ecological zones, ZVFS ZSO are expanding in population while the remaining zones are in balance.

Keywords: Haplotype, agro-ecological zone, demographic parameters, C. maculatus, genetic diversity, expansion, balance, Senegal

Introduction
Organisms can only survive if their DNA desoxyribonucleic acid) is carefully replicated and protected from chemical and physical alterations that could change its coding properties. Thus life and biodiversity result from a subtle balance between mutations and repairs.
Mutations, depending on their nature, have various effects on the phenotype: some have no impact, while others cause the appearance of new variations of a character. It is therefore the mutations that induce the great genetic variability and thus the great diversity of hereditary traits. They introduce a genetic variability necessary for evolution generated by the effect of four evolutionary forces: selection, genetic drift, mutations and migration.
Genetic diversity is a measure quantifying the extent of genetic variability within a population (Hughes *et al.*, 2008) [11]. According to Frankham *et al* (2002) [7], it is the raw material on which selection acts to allow adaptation to environmental changes, and thus evolution. It is at the origin, with the environment, of the variation of observable characteristics, such as shape, size... It thus allows species to adapt to a constantly changing environment, to resist parasites.... Genetic diversity is expressed by the frequencies of different alleles at a locus. According to Ellegren and Galtier (2016) [4]. genetic diversity is generated by the appearance and disappearance of genetic variants called alleles. Within a species, different alleles coexist for a given gene (versions of that gene). These alleles have appeared over time as a result of germline mutations that have spread through the populations. Note also that their maintenance 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 (2008) [14]. The term haplotype corresponds to a nucleotide sequence, which 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.
Thus, in order to highlight the circulation of haplotypes within *Callosobruchus maculatus (C. maculatus)* populations in the agro-ecological zones of Senegal, we set three objectives in this article:
To identify the different haplotypes encountered in the agro-ecological zones of Senegal, to determine their distribution and the demographic evolution of the different populations studied.

Materials and Methods
1. Sampling
Samples were taken throughout Senegal, which allowed us to cover all agro-ecological zones except the niayes where cowpea is rarely grown. Indeed, for each zone, samples from different localities were collected. Samples were collected during the post-harvest period from farmers or from the market in each locality. The samples were clearly identified with the name of the locality of origin. These samples, weighing a minimum of 250g, were taken to the rearing laboratory where they were transferred into well

aerated jars that did not allow the insects to escape. The rearing was done in the laboratory under ambient conditions until the emergence of the adults of *C. maculatus* with regular monitoring. Following emergence, the insects were collected and placed in 96° alcohol. The tubes containing the specimens of *C. maculatus* were placed in the refrigerator for a good conservation. These specimens were used for genetic studies. Prior to the work for the genetic studies, the identified specimens were coded according to an alphanumeric code defined by the first letters of the binomial name of the species (C for *Callosobruchus* and m for *maculatus*), followed by the first letter of the name of the sampled locality after which comes a second letter indicating the second name of the locality which is finally followed by the sequence number indicating the individual (Example: Code CmCd1; *Callosobruchus* (C) *maculatus* (m) Carrefour (C) diaroumé (d) individual (1). For the other samples, we simply took the name of the locality (Table 1).

Table 1: sampling summary

Agro-écological zones	Sampled localities	Sample codes	Number of zindividuals	Geographical coordinates	
				Latitude	Longitude
Groundnut basin zone (ZBA)	Fatick	Cmfatick	10	14°20'22''N	16°24'40''W
Casamance zone (ZC)	Carrefour diaroumé	CmCd	07	13°03'31''N	15°38'20''W
	Diannah malary	CmDm	07	12°50'15''N	15°15'07''W
Eastern Senegal zone (ZSO)	Tamba	Tamba	12	13°46'14'' N	13°40'2''W
Senegal river valley zone (ZVFS)	Fouta	Fouta	09	16° 32'01''N	16°08'19''W
Sylvo-Pastoral zone (ZSP)	Barkédji	CmBb	09	15°16'40''N	14°52'02''W
	Koki	Koki	10	15°30'20''N	15°59'30''W
Total			64		

The individuals coming from the samples of the same area form a population. This corresponds to a total of 7 populations. The size of the populations studied is a function of the number of individuals taken per sample; this number varies according to the locality.

2. DNA analysis

2.1 Cytochrome B (Cyt-B)

One of the most consistently used genes in studies of molecular evolution and species structuring of insect pests is the mitochondrial Cyt-B gene [22, 21].

The mtDNA is haploid (N), not recombinant [20]. It is transmitted almost exclusively through the maternal body. The mtDNA is more suitable for the demonstration of specific variability than nuclear DNA. The gene encoding Cyt-B is widely used in molecular phylogeny [2, 15] and also in population genetics [10].

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 [1].

2.2 Extraction of DNA from C. maculatus

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 *C. maculatus* 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.

2.3 DNA amplification by polymerase chain reaction (PCR)

In the PCR amplification of DNA, the forword and reverse primers used are 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 one minute at 47°C for hybridization and extension of the primers or elongation for a time of one minute at 72°C and (iii) comes a final elongation at 72°C for 10 minutes.

2. 4 Sequencing

DNA sequencing consists in determining the sequence of nucleotides of 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.

Genetic Analysais

1. Alignments, cleaning and correction of sequences

According to Swofford *et al* [24], sequence alignment is important in determining whether or not sites are similar. The sequences were aligned as a whole using the Bio Edit ver. 7.2.5 softwares [25]. 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 [26] which is part of the so-called global alignment methods.

2. Demographic evolution

The demographic history of *Callosobruchus maculatus* in Senegal was studied by performing demogenetic tests (Tajima's D, Fu's FS, sum of squares of deviations (SSD), Raggedness index (rg) and Ramos' R2 were determined) and by analyzing the mismatch distribution.

2.1 Demo-genetic tests

Indices such as Tajima's D and Fu's FS [23]; [8] were used to test the deviation from the neutrality hypothesis by DNASp 5.10.01 software [19]. These indices are known to be sensitive to departures from mutation-drift equilibrium due to changes in population sizes and selection [16]. Fu's FS test compares the average number of pairwise differences with the number of haplotypes (h) in the population. Tajima's D is based on the difference between the average number of pairwise differences and the number of variable sites.

Under the 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 non-negative D values suggest recent population expansion while the opposite suggests selection.

Complementarily, Ramos' R2 statistic [17], based on the differences between the number of singletons and the average nucleotide differences, was calculated with DNASp 5.10.01 [19]. Ramos-Onsins and Rozas [17] 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 sum of squares of deviations (SSD) between the observed and expected distributions as well as the Raggedness index (rg) were calculated and determined under the assumption of an expanding population by Arlequin V3.1 [5].

2.2 Mismatch Distribution Analysis

The mismatch distribution analysis or the distribution of the observed number of differences between pairs of haplotypes [18] was estimated by DNASp 5.10.01 [19]. The expected values were constructed assuming a constant population size: according to Rogers & Harpending [18], a recent rapid population growth is characterized by a unimodal distribution while a multimodal distribution characterizes a population at demographic equilibrium.

Results

1. Haplotypes

The 64 Cyt-B gene sequences we studied yield 20 haplotypes, 10 of which are unique (H6, H7, H8, H9, H10, H13, H15, H16, H17 and H18). However, there are three major haplotypes (H19, H1 and H4) composed of 10, 11 and 13 individuals respectively. The populations of Koki and Tamba are the most homogeneous. Indeed, we note that all the individuals of Koki belong to the same haplotype H4 where they are in the majority except for one (koki10) which is of haplotype H15. Haplotype H19 is made up only of individuals from Tamba. Haplotype 1 is made up mainly of individuals from Fouta.

Table 2: Number and composition of haplotypes

Haplotypes	Work force	Individuals
H1	10	CmBb1 CmBb4 CmBb10 CmFatick2 Fouta1 Fouta2 Fouta5 Fouta7 Fouta8 Fouta9
H2	07	CmBb2 CmBb7 CmBb9 CmCd1 CmCd4 CmCd5 CmFatick3
H3	02	CmBb5 CmBb8
H4	13	CmBb6 CmDm1 CmDm4 CmDm6 Koki1 Koki2 Koki3 Koki4 Koki5 Koki6 Koki7 Koki8 Koki9
H5	04	CmCd2 CmCd3 CmCd8 Fouta3
H6	01	CmCd9
H7	01	CmDm2
H8	01	CmDm3
H9	01	CmDm5
H10	01	CmDm9
H11	03	CmFatick1 CmFatick4 CmFatick10
H12	02	CmFatick5 CmFatick8
H13	01	CmFatick6
H14	02	CmFatick7 CmFatick9
H15	01	Koki10
H16	01	Fouta4
H17	01	Fouta6
H18	01	Tamba1
H19	09	Tamba2 Tamba3 Tamba4 Tamba5 Tamba6 Tamba8 Tamba9 Tamba11 Tamba12
H20	02	Tamba7 Tamba10

These haplotypes can be considered according to their distribution within the agro-ecological zones. Thus, we noted that the Casamance zone (ZC) has the greatest number of haplotypes; eight (08) in total out of the 14 individuals that constitute its population studied here. This is followed by the Peanut Basin Zone (PBZ) which has six haplotypes out of only 10 individuals. The Eastern Senegal zone has the lowest number of haplotypes (only four out of twelve individuals).

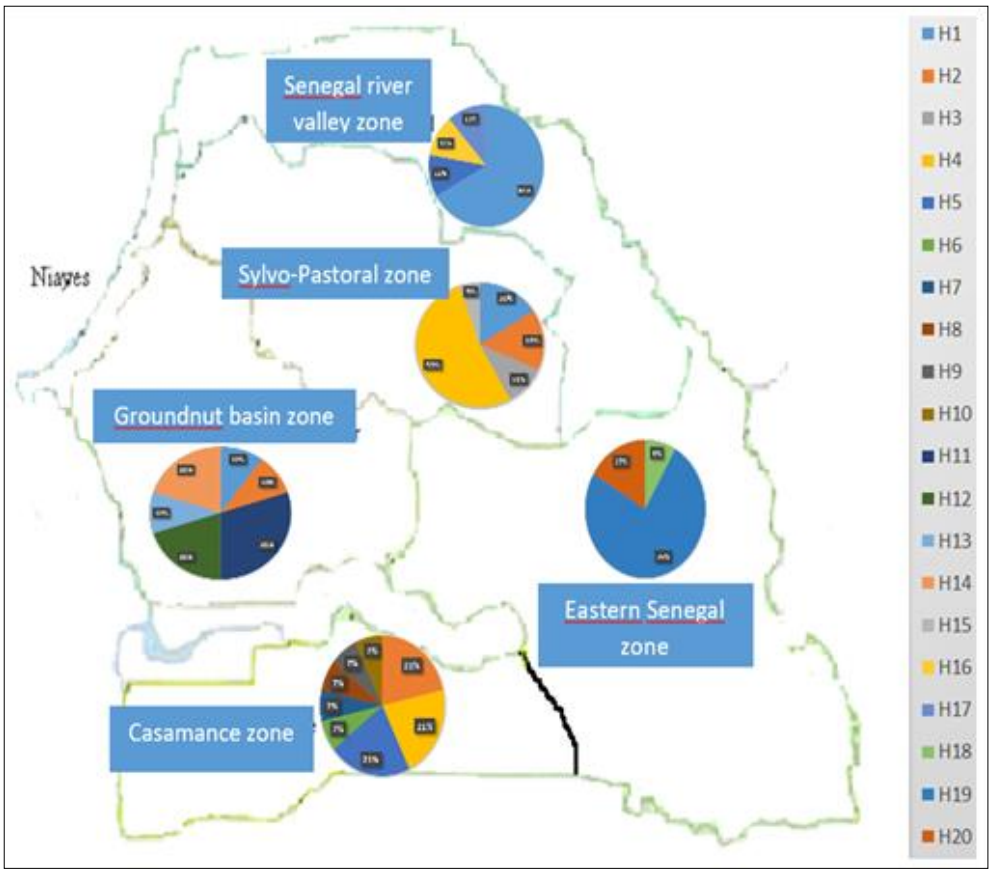


Fig 1: Distribution of haplotypes according to agro-ecological zones

Population growth

1. Neutrality Indices

Neutrality indices are calculated by population, by area and globally. Taking the total population, we get Fu's FS that is significantly negative and Tajima's D that is positive and not significant (P-value > 0.10). Ramos' R2, rg and SSD are all positive but not significant. (Table3).

Table 3: Demographic Parameters of the total population of C. maculatus.

	Fs de Fu	***D de Tajima***	***Ramos'R2***	***rg***	***SSD***
Values	-4,597	1,451	0,539	0,629	0,100
P-values	0,006	P>0,10	0,09911	0,02143	0,329

The populations taken one by one showvalues of Tajima's D and Fu's FS that are positive and non-significant for the populations of Barkédji and Carrefour diaroumé, positive D and negative FS values but all non-significant for those of Diannah and Fatick. Populations from Fouta, Koki and Tamba have negative D and FS values but only significant for Fouta. Thus, only the populations from Barkédji and Carrefour had positive Fu FS. The populations each have positive and insignificant values of SSD, rg and R2 of Ramos except for the Carrefour diaroumé population which has a significant SSD. (Table 4).

Table 4: Neutrality indices by population.

Populations Indices	Barkédji	Carrefour diaroumé	Diannah malary	Fatick	Koki	Fouta	Tamba
D de Tajima P-value	0,397 0,698	1,439 0,945	0,863 0,825	0,205 0,619	-1,112 0,180	-1,610 0,038	-0,850 0,248
Fs de Fu P-value	2,252 0,874	2,314 0,889	-0,4945 0,319	-0,097 0,473	-0,339 0,146	-1,283 0,039	-0,725 0,077
SSD P-value	0,093 0,080	0,167 0,050	0,024 0,660	0,072 0,110	0,331 0,080	0,001 0,950	0,011 0,370
Raggedness (rg) P-value	0,234 0,070	0,327 0,150	0,075 0,810	0,114 0,470	0,400 0,270	0,049 0,990	0,167 0,460
Ramos'R2 P-value	0,551 0,167	0,521 0,187	0,520 0,186	0,502 0,161	0,508 0,162	0,533 0,166	0,549 0,151

According to the agro-ecological zones, only the Senegal River valley zone has a Tajima D and Fu FS that are significantly negative. The Eastern Senegal zone has a Tajima D and Fu FS that are negative but not significant. The Sylvo-pastoral zone has indices that are positive and not significant. The Casamance and Groundnut Basin zones have positive D and negative and insignificant FS. All zones had negative Fu FS except the Sylvo-pastoral zone. The agro-ecological zones also all have positive and non-significant Ramos R2 values. Their SSD and rg are also positive and insignificant except for the Sylvo-pastoral zone which has a significantly positive SSD and the Casamance zone, which has a significantly positive rg. (Table 5).

Table 5: Neutrality indices by agro-ecological zone

Zones/ Indices	ZSP	ZC	ZBA	ZVFS	ZSO
D de Tajima P-value	1,282 0,930	1,067 0,884	0,205 0,612	-1,610 0,038	-0,850 0,225
Fs de Fu P-value	3,104 0,911	-0,792 0,314	-0,097 0,479	-1,283 0,044	-0,725 0,076
SSD P-value	0,094 0,020	0,045 0,110	0,072 0,170	0,001 0,950	0,011 0,360
Raggedness (rg) P-value	0,146 0,080	0,066 0,0400	0,114 0,400	0,049 0,970	0,167 0,550
Ramos'R2 P-value	0,5250,132	0,571 0,141	0,502 0,161	0,533 0,166	0,549 0,151

2. Mismatch distribution

The total population shows a mismatch curve that is multimodal. (Figure 2).

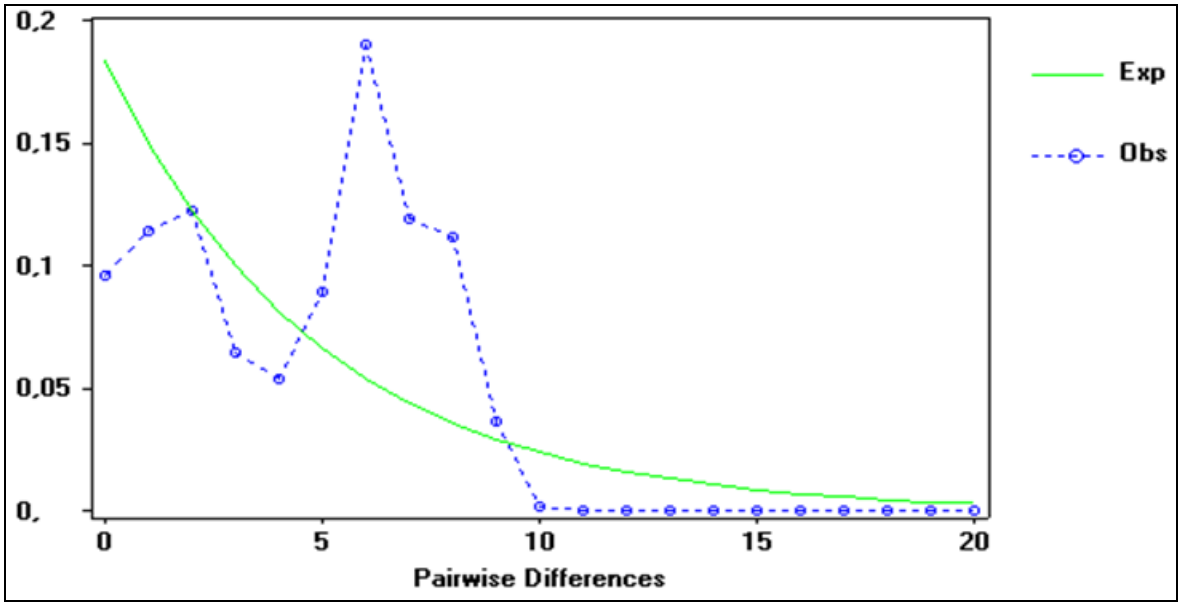
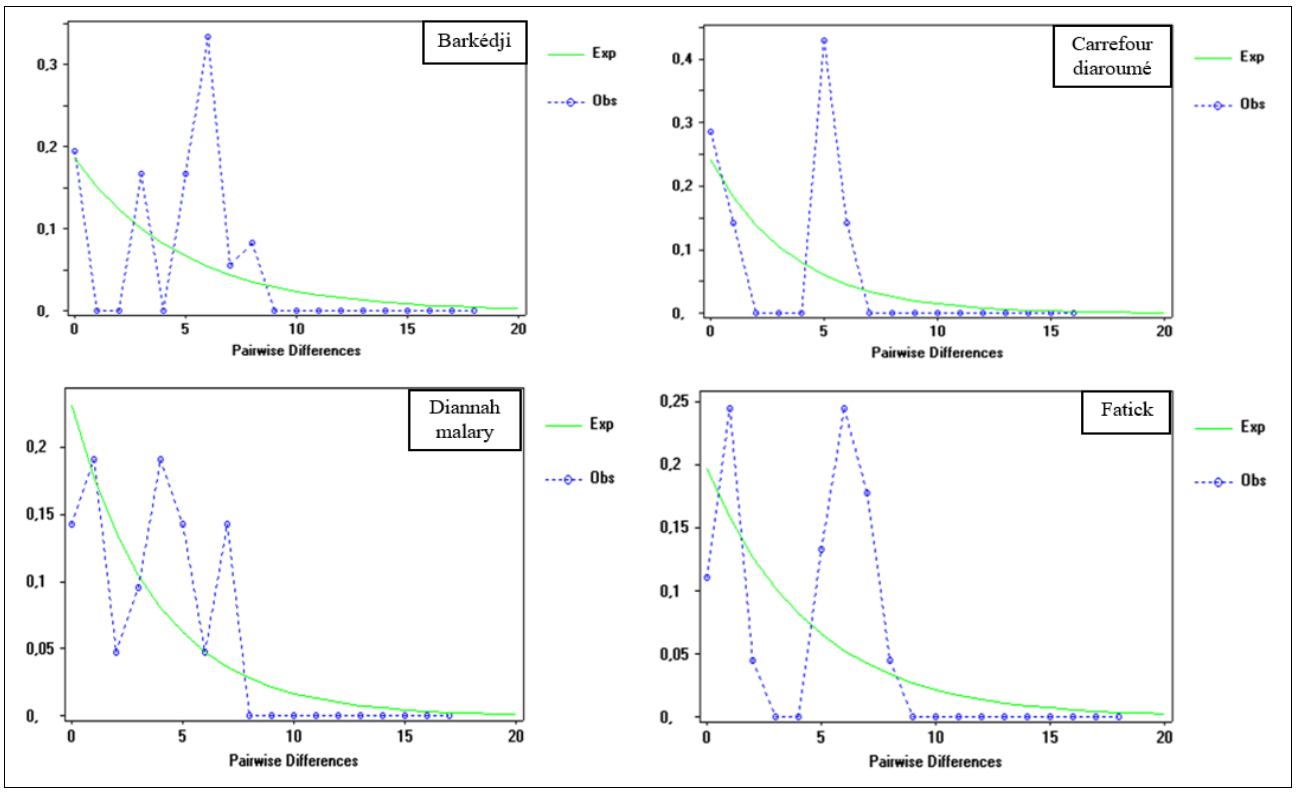


Fig 2: Mismatch curves for the total population showing expected and observed frequencies (solid and dashed lines respectively).

The mismatch curves for the populations from Fouta, Koki, and Tamba are all unimodal and show almost no difference between expected and observed results. In contrast, the populations of Diannah malary (CmDm), Fatick (CmFatick), Barkedji (CmBb), and Carrefour diaroumé (CmCd) have multimodal mismatch curves.



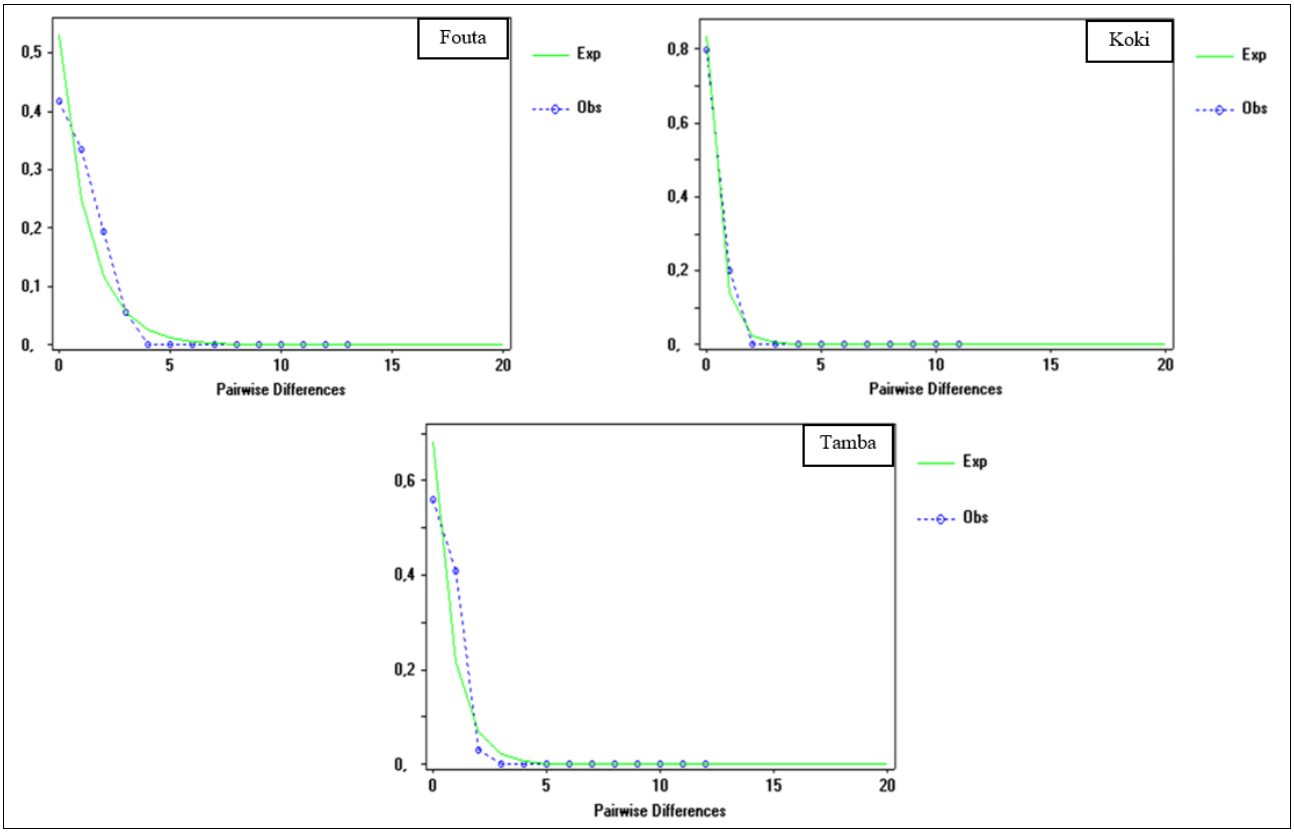
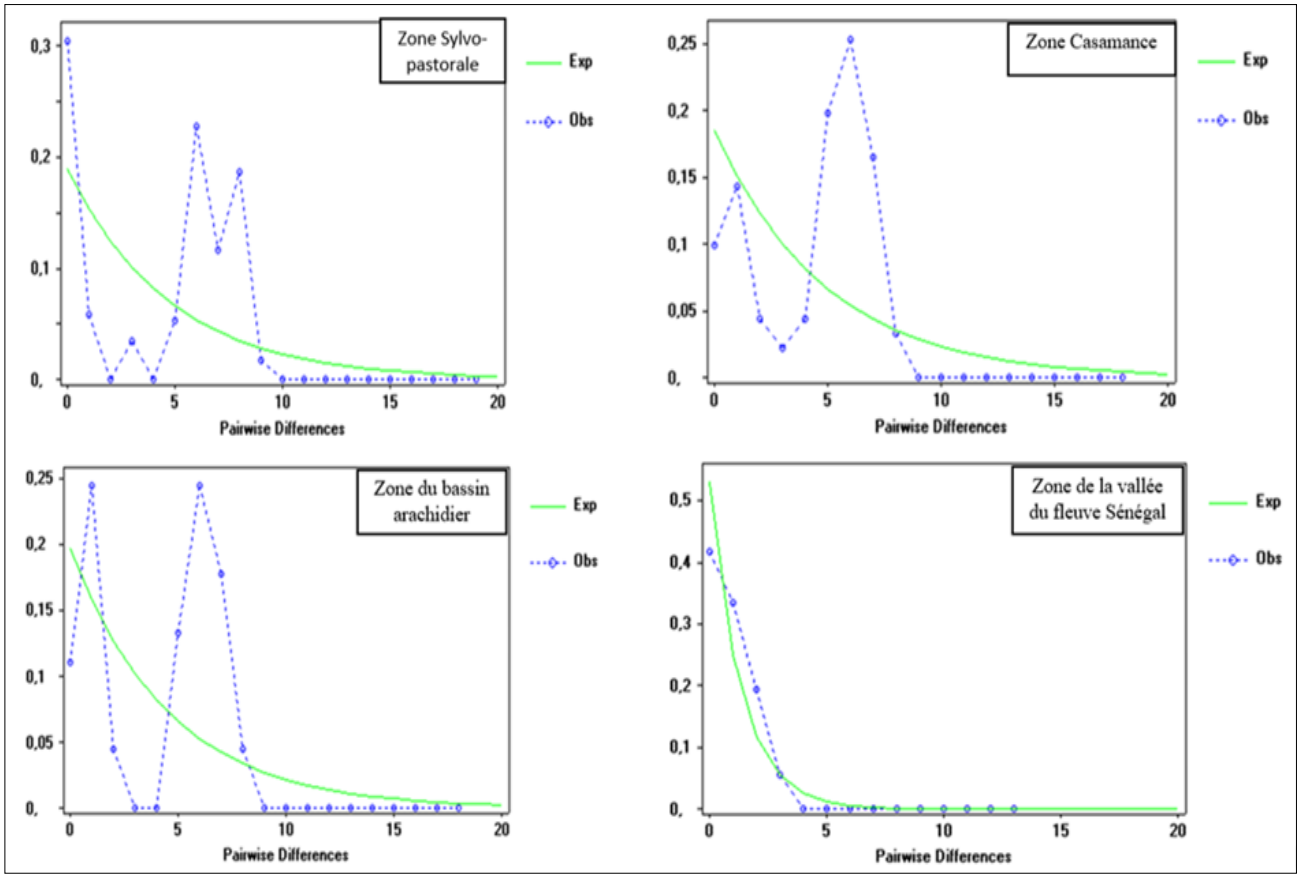


Fig 3: Mismatch distribution curves of the sampled populations showing expected and observed frequencies (solid and dashed line respectively).

The mismatches taken by agro-ecological zone are multimodal for the Groundnut Basin, Casamance and Sylvo-pastoral zones but unimodal for the Eastern Senegal and Senegal River Valley zones.



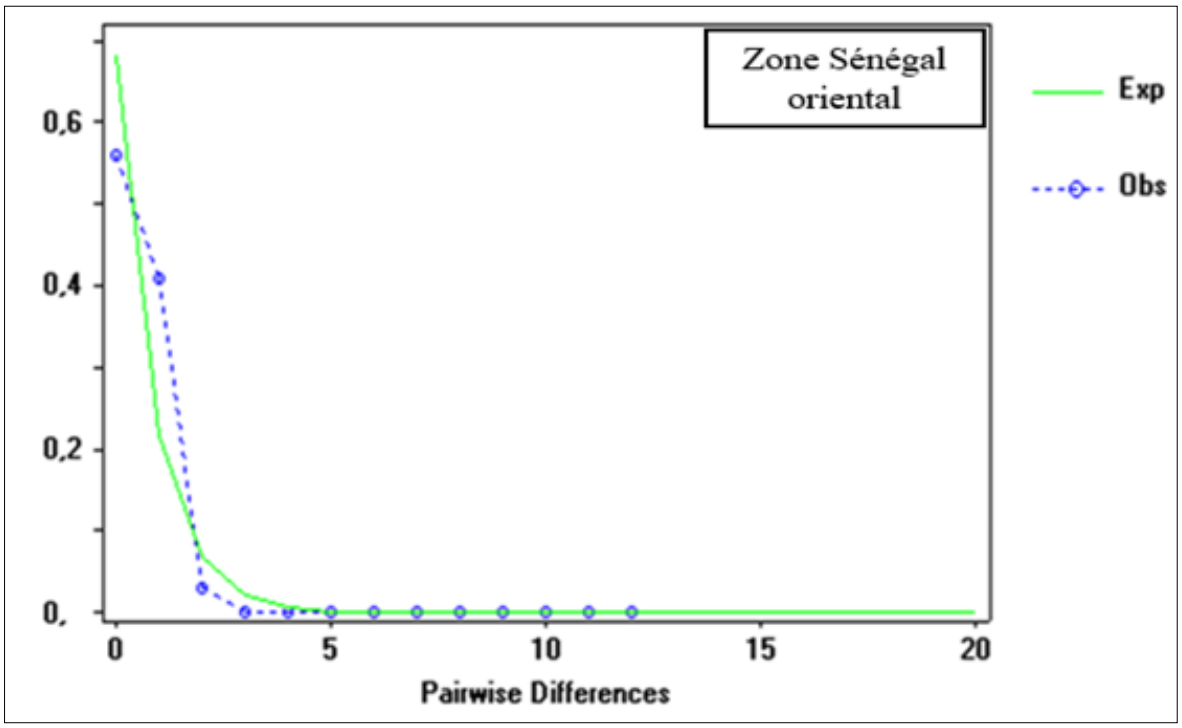


Fig 4: Mismatch distribution curves for populations sampled by agro-ecological zone showing expected and observed frequencies (solid and dashed lines respectively), the number of nucleotide differences per pair is plotted on the x-axis, the y-axis corresponds to pairs of individuals

3. Haplotype network

The network shows three major haplotypes H4, H19 and H1. The H4 haplotype has the largest population. However, we find that the H19 haplotype is the ancestral haplotype with two large branches from which the H4 and H1 haplotypes are connected. Between H19 and H2 we have a mutation step as well as between H7 and H4. The haplotypes H6, H10, H13, H14, H16, H17 and H20 are peripheral. Among these, the individual ones are more numerous (H6, H10, H13, H16 and H17).

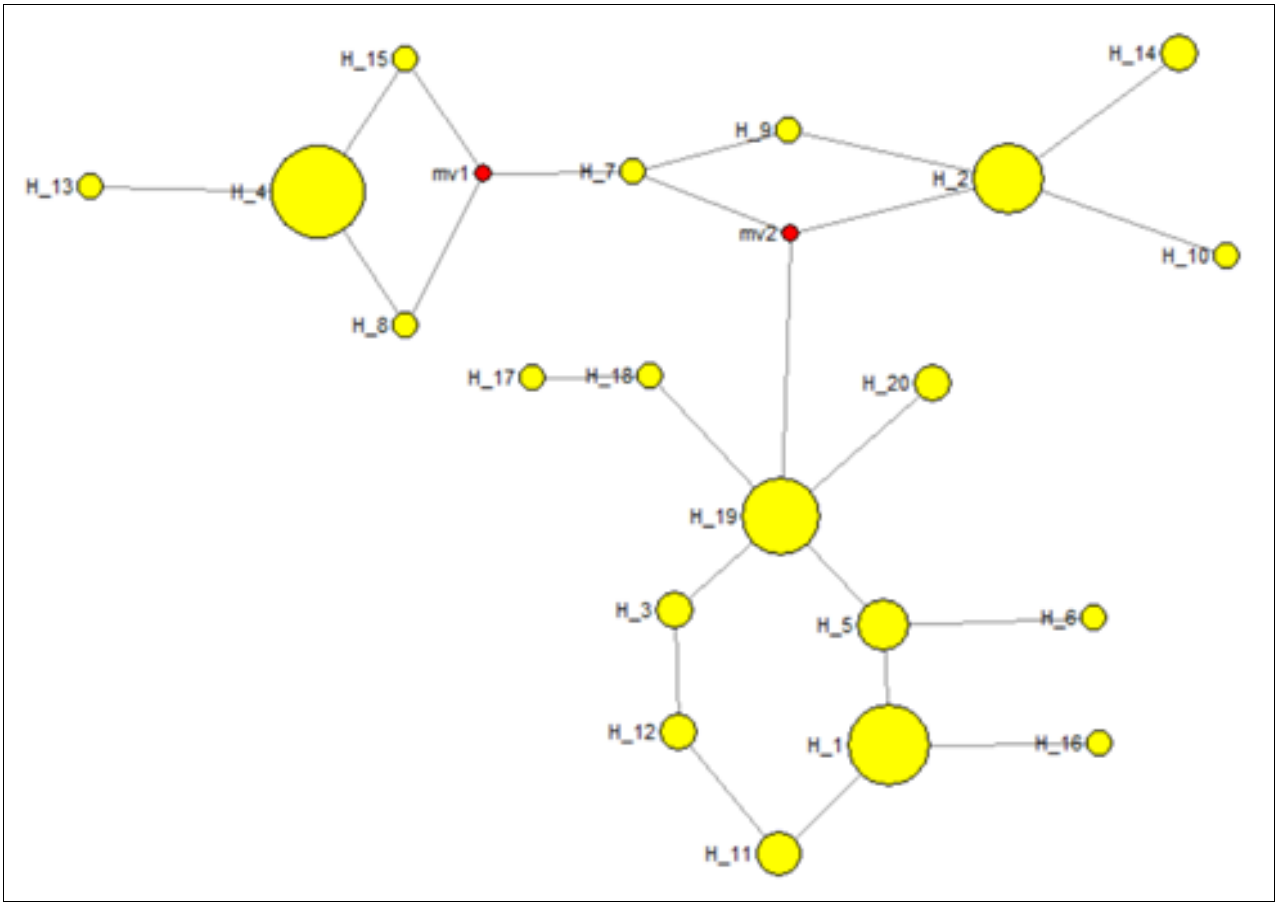


Fig 5: Haplotype network

Discussion

Insect pests of cowpea stocks, *Callosobruchus maculatus* populations have been the subject of various studies, few of which are devoted to the genetic aspect.

The objective of this work is to determine the haplotypes circulating in populations of this insect in the different agro-ecological zones of Senegal using the Cyt-B mitochondrial gene.

Analysis of the cytochondrial gene sequences obtained showed that the populations of C. maculatus studied contained different haplotypes. At least 20 haplotypes of cowpea bruchid circulate in the different agro-ecological zones sampled. The level of genetic variation is therefore high with 20 haplotypes out of 64 sequences analyzed. These results are comparable to those obtained by Kébé [12]. Mutations thus induced the appearance of distinct haplotypes. This resulted in a fairly high number of individual haplotypes; 10 in total. In general, we can see a connectivity between the different populations, which means that there has been a gene flow between them. Grant and Bown [9], considering the levels of diversity detected through these two indices, argue that under conditions of rapid expansion, haplotypic diversity grows faster than nucleotidic diversity, leading to a large number of single haplotypes.

The majority haplotypes H1, H4, H19 and to some extent H7, show a low level of diversity. These haplotypes have circulated within zones but very little from one zone to another since the individuals constituting them are mostly from the same locality. In these areas, genetic diversity is not significant. This observation could reflect a past bottleneck, followed by a rapid expansion of the population and an accumulation of mutations. This indicates that these populations are not in a mutation-drift equilibrium state, i.e. their effective size has not remained constant over time.

These results indicate a signal of a stable total population with large effective size or admixture from populations that have been isolated from each other [13]. This is also consistent with the finding of Dia *et al* [3] on other populations of beetles that depend on stored or traded commodities.

In the Silvo-pastoral zone (SPZ), one haplotype (H4) circulates between the two populations studied, indicating inter-population mixing. Haplotypes H1 and H2 are the most shared; each is found in three of the five zones considered.

Haplotype H19 is a central haplotype from which all the others originate and it is made up only of individuals from Tamba. This is consistent with our idea of a unidirectional circulation of cowpeas from Tamba to the other localities. This is also an explanation for the non-diversification of bruchid populations in this zone, as well as the phenomenon of varietal monoculture.

The demogenetic parameters reveal that the total population of *C. maculatus* has undergone a recent demographic expansion.

The structure of the network appears complex with many infrequent and not very divergent haplotypes, with a fairly large number of peripheral individual haplotypes. The network is therefore not well structured. In general, we can see connectivity between the different populations, which means that there has been gene flow between them. This aspect of the structure is consistent with a non-significant Tajima D value and a significantly negative Fu FS. The presence of a central haplotype (H19) provides information on the link between the different populations. Indeed, we can say that it is from the Tamba population that the others derive and begin to be singled out according to their area of existence. This observed structuring, which is generalist, could be explained by the fact that *C. maculatus* behaves like a panmictic unit. This is in line with the work of Kébé [12]. This interbreeding pattern is accentuated by trade.

According to Excoffier *et al* [6], a negative Tajima D could correspond to a demographic expansion. Thus, the populations of the localities of Fouta, Koki and Tamba that have negative Tajima D and Fu FS are undergoing demographic expansion, as are those of the agro-ecological zones of the Senegal River valley and eastern Senegal (ZVFS and ZSO). This expansion is even confirmed by the other demographic parameters calculated such as SSD, Raggedness irregularity index (rg). On the other hand, the remaining populations are in equilibrium. This is confirmed by the mismatches. Indeed, the mismatch curves reveal two demographic patterns: a demographic expansion for the populations of Fouta, Tamba and Koki, and populations in equilibrium such as those of Barkédji, Carrefour diaroumé, Diannah malary and Fatick. Depending on the area, the populations of the Senegal River Valley and the Eastern Senegal area are expanding, while those in the other areas are in balance. The population balance noted is confirmed by the values of the SSD and the rg for the locality of Carrefour diaroumé and for the agro-ecological zones of Casamance and the Sylvo-pastoral zone. On the other hand, the SSD and RG for the agro-ecological zone of the groundnut basin and the localities of Diannah Malary, Barkédji and Fatick confirm a demographic expansion. It should also be noted that the Ramos' R2 do not confirm any population status for either the localities or the agro-ecological zones.

Conclusion

Subject to further study, with a larger number of samples, a number of ideas can be retained. The recorded results allow us to highlight 20 haplotypes out of the 64 sequences analyzed. Among these 20 haplotypes, 10 are individual. This corresponds to a low circulation of haplotypes within the populations studied. The demographic parameters reveal expanding populations and populations in equilibrium. However, all these populations have an ancestral link with the individuals of Tamba. However, could the results obtained reflect a well-defined genetic diversity and structuring?

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