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Genetic diversity of *Callosobruchus maculatus* F. populations in Senegal according to the two morphotypes: Flight morph and flitghless morph

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Abstract

In developing countries, cowpea or *Vigna unguiculata* L. (Walp) is one of the staple foods of the population with cowpea seeds that are rich in proteins, carbohydrates and vitamins. Indeed, cowpea is attacked by a wide spectrum of insect pests, the Bruchidae Coleoptera, including *Callosobruchus maculatus* Fabricius. The conséquence of these attacks is the qualitative and quantitative decrease of the harvested or stored seeds due to the emergence of the flight morph from the flightless morph. The present work aims to determine the genetic diversity between the two morphotypes of *Callosobruchus maculatus in* Senegalese populations in order to develop more efficient control methods against this insect pest of cowpea seeds in storage. To carry out this work, we sampled twenty-nine (29) individuals constituting the flight morph population and nineteen (19) individuals forming the flightless morph population. After extraction, purification and DNA recovery using the Standard Qiagen method (Qiagen Kit), the Cytochrome B gene was amplified to obtain the studied sequences. However, flightless morphotypes are more monomorphic than the flight morph populations which are then more polymorphic because of a high rate of mutation per transition. On the other hand, the high rate of mutation per transversion in the flightless morph population could be favorable to a change of aminoacids and corresponding protein such as enzymes involved in metamorphosis. Indeed, this genetic diversity associated with haplotypic diversity could be a source of weak to moderate differentiation and this result was confirmed by the inter-population genetic distance which is greater than the intra-population genetic distance. This result would be partly due to environmental pressures leading to a weak genetic structuring between the two morphotypes according to the AMOVA values which present a significantly high percentage of variance. The neutrality tests and the Bayesian tree confirm this differentiation between the two morphotypes linked to an important gene flow within the total population. This suggests that some heterozygous flightless morph individuals have genetic predispositions to become flight morph individuals with the evolution of environmental conditions combined with the demographic and the maternal effect related to the female's choice of spawning.

Keywords: *Callosobruchus maculatus*, morphotypes, flight morph, flightless morph, Cytochrome B, mutation, transition, transversion, differentiation and genetic structuring, environment

Introduction

Cowpea or Vigna ungniculata (L) Walp is the second largest legume after groundnut in the traditional Senegalese farming system. It's a valuable source of protein whose high rate (22 to 24%) and excellent quality make it an important part of the nutritional balance of rural populations, particularly in the fight against protein deficiency in children [1]. In addition to its interest in human food in a perspective of food self-sufficiency and crop diversification, cowpea can constitute a food of choice for live-stock because of the quality of its leaves. However, cowpea production, already affected by climatic hazards, is limited by losses caused by Bruchidae insects, including Bruchidius atrolineatus and Callosobruchus maculatus, which make post-harvest preservation very difficult. Indeed, the larval stages of these insect pests develop inside the seeds and consume the reserves contained in the cotyledons leading to considerable losses on the stored seeds [7]. The objective of this study is to determine the genetic diversity between the two morphotypes (flight morph and flightless morph populations) of Callosobruchus maculatus through a molecular marker such as Cytochrome B in order to elaborate an integrated and reasoned control in order to reduce or avoid the losses caused by this pest on cowpea crops.

Material and methods

1. Sampling

Cowpea seeds harvested after overwintering are placed in jars and stored at room temperature. The insects that emerge are immediately collected and preserved in alcohol (96%). The individuals of the species studied come from different localities in Senegal and are constituted in populations according to their ability or not for flight in two morphotypes: flight morph and flightless morph. Twenty-nine (29) individuals were sampled to constitute the flight population and nineteen (19) individuals were sampled to constitute the flightless morph population.

2. DNA Extraction, PCR and Sequencing of Cytochrome B

After extraction of genomic DNA from C. maculatus individuals according to the standard extraction protocol DNeasy Tissue kit from QIAGEN, amplification of the Cytochrome B gene is performed using the following primers: CB1 (5'TATGTACTACCATGAGGACAAATATC and CB2 (5'ATTACACCTCCTAATTTATTAGGAAT), with a hybridization temperature of 47°C, in the presence of Mg2+ ions whose concentration can be readjusted in order to optimize the results.

The three PCR steps are performed in an Eppendorf-type thermal cycler with a total reaction volume of 25 µl under the conditions describedby [8]. Sequencing is out sourced to a company in South Korea. From each of the 2 primers, sequencing based on the use of particular nucleotides called did deoxyribonucleotides is initiated according to the method adopted by [14]. The results of the sequencing are received by email after about a week in text file format.

3. Genetic analysis

The number of polymorphic sites, the number of parsimony informative sites, the rate of transitions/transversions (R) and the average number of nucleotide frequencies were calculated using MEGA 7.0.14 software [17]. To determine the genetic variation of C. maculatus, the number of haplotypes as well as the haplotypic and nucleotide diversities were calculated using DnaSP software version 5.10.01 [9]. Genetic differentiation FST [19] per population pair was calculated using Arlequin software v3.5.1.2 [4], as well as analysis of molecular variance (AMOVA) to determine the genetic structuring of populations. The closer the FST approaches the value of 1, the more genetically structured the populations are among themselves. The genetic distance (d) between populations taken two by two was calculated in MEGA, using the 2-parameter model (K2P). Phylogenetic relationships between C. maculatus populations were estimated using Bayesian inference with MrBayes v. 3.1 software [6]. Reconstructions were rooted with a homologous sequence of Caryedon serratus (Ca ser). The algorithm [18] was used to estimate the network of haplotypic or allelic relationships. The network was constructed using Network software version 4.6.1.3. The demographic history of C. maculatus of these two forms was studied by calculating Tajima's D [16] and Fu's Fs test [5] indices and analysing the distribution disparity (mismatch distribution). Recent rapid population growth is characterised by an unimodal distribution, while a multimodal distribution characterises a population in demographic equilibrium [13]. Distribution quality indices such as the sum of squared deviations (SSD) between the observed and expected distributions and the Raggedness Irregularity Index (Rag) of the observed distribution of mismatched classes were calculated under the assumption of an expanding population using the Arlequin software.

Results

1. Genetic polymorphism and variability

After alignment and correction of the 48 mitochondrial cytochrome B gene sequences, the data base consists of 393 sites without gaps or deletions. Analyses of genetic variability showed an average of 78.62% conserved sites and 21.37% variable sites, of which 7.63% were informative sites in parsimony for the total population. The R mutation rate is 1.5. According to the flight and flightless morphotypes, we noted a considerable increase in the rate of conserved sites from 84.47% to 92.62% and a strong decrease in the rate of variable sites from 7.38% to 15.52% compared to the total population (see table I).

Table 1: Distribution of genetic variability according to the two morphotypes

	Total population	Flight morph	Flight less morph
Conserved sites	78.62%	84.47%	92.62%
Polymorphic variable sites	21.37%	15.52%	7.38%
Informative sites	7.63%	3.81%	4.32%

Considering both morphotypes, the R mutation rate is 1.5 for the total population but it varies rapidly according to flight ability between 0.699 for the flight morph and 5.787 for the flightless morph. However, the R mutation rate is low for the flight morph population and very high for the flightless morph population. In fact, in the total population, 65.63% of mutations are transitions, where as 34.38% of mutations are transversions favoring in most cases a change of amino acid and protein structure. The transversion rate is significantly higher in flight morph than in flightless morph (Table II).

Table 2: Nature and mutation rate according to the two population forms.

	Total population	Flight morph	Flighless morph
Transition rate (Ts)	65.63	48.25	88.89
Transversion rate (Tv)	34.38	51.74	11.12
Mutation rate R=Ts/Tv	1.5	0.699	5.787

This important genetic variability could be at the origin of the emergence of the flight morph individuals. Indeed, the low R mutation rate compared to the total population is partly justified by the high transversion rate resulting in changes in amino acids and protein structure. The haplotypes h and the average number of nucleotide differences between k sequences are relatively high. Nucleotide diversity indices Pi and haplotype diversity Hd are determined first within each population and then for all individuals as a whole (Table III). The results reveal the same trend with a high haplotypic diversity close to 1 and a very low nucleotide diversity close to 0 in the two different populations compared to the total population. Further-more, considering both morphotypes, all diversity indices are higher for the flight morphotypes and low for the flightless morphotypes.

Table 3: Genetic diversity indices

Parameters Populations	h	Hd	Pi	k
Flight morph	16	0.916±0.0005	0.01978±0.00001	7.773
Flightless morph	10	0.825±0.0015	0.01497±0.00004	5.883
Total population	25	0.937±0.0003	0.02031±0.0037	7.982

Genetic distances

The intrapopulation distance is 0.015 and it’s lower for the flightless morphotype while it is 0.021 for the flight morphotype. However, the genetic distance is relatively higher (0.023) between the two morphotypes (Table V).

Table 4: Intra- and inter-population genetic distances

Distances génétiques			
	Intra population	Inter-populations	
		Flight morph	Flightless morph
Flight morph	0.021	----	
Flightless morph	0.015	0.023	----

Genetic structuring

Approximately 21.635% of the total genetic variability is explained by the genetic diversity between the two flight and flightless morphotypes. The rest of the variability is explained by genetic diversity between individuals (see table VI).

Table 5: Analysis of molecular variance

Source of variation	Degree of freedom (d. f.)	Sum of squares	Variance components	Percentage change
Between populations	1	25.808	Va=0.9709	21.635%
Between individuals	46	161.775	Vb=3.5168	78.365%
Total	47	187.583	4.4878	100%

Demographic trends

1. Demogenetic tests

The analysis results reveal negative and non-significant values of Tajima's D and Fu's Fs tests for the flight morphotype. On the other hand, for the flightless morphotype, Tajima's D values are positive and non-significant ($p < 0.05$) and Fu's Fs values are negative and non-significant. however, the total population shows negative and significant values for Tajima's D ($p > 0.05$) but not significant for Fu's Fs (see Table VII)

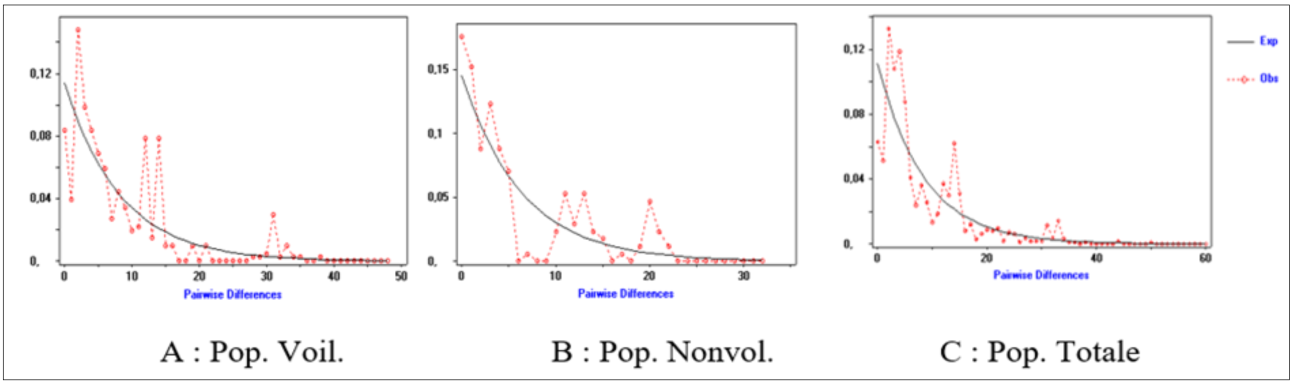
Table 6: Indices of demographic change

Populations Parameters	Total population	Flight morph population	Flightless morph population
Tajima’s D p-value	-1.5202* 0.05	-1.8891 0.130	0.1514 0.105
Fu’s Fs p- value	-1.3228 0.332	-2.1235 0.2080	-0.5220 0.4320

* Significant p-value ($p < 0,05$)

Mismatch distribution

The two mismatch distribution curves represent observed (red) and expected (black) nucleotide frequencies under the assumption of population expansion based on nucleotide differences between sequence pairs. The observed number of nucleotide differences between paired haplotypes produced an unimodal distribution on each of the graphs revealing a recent rapid growth in the populations of both C. maculatus morphotypes and the total population (see Figure 1).



A : Pop. Voil. B : Pop. Nonvol. C : Pop. Totale

Fig 1: Distribution of the number of nucleotide differences between paired haplotypes of flight (A) and flightless morphotypes (B) and the total population (C) of C. *maculatus.*

The irregularity index (R) and the sum of squared deviation (SSD) are positive and insignificant for each population (Table VIII). This confirms the absence of gap between the two curves

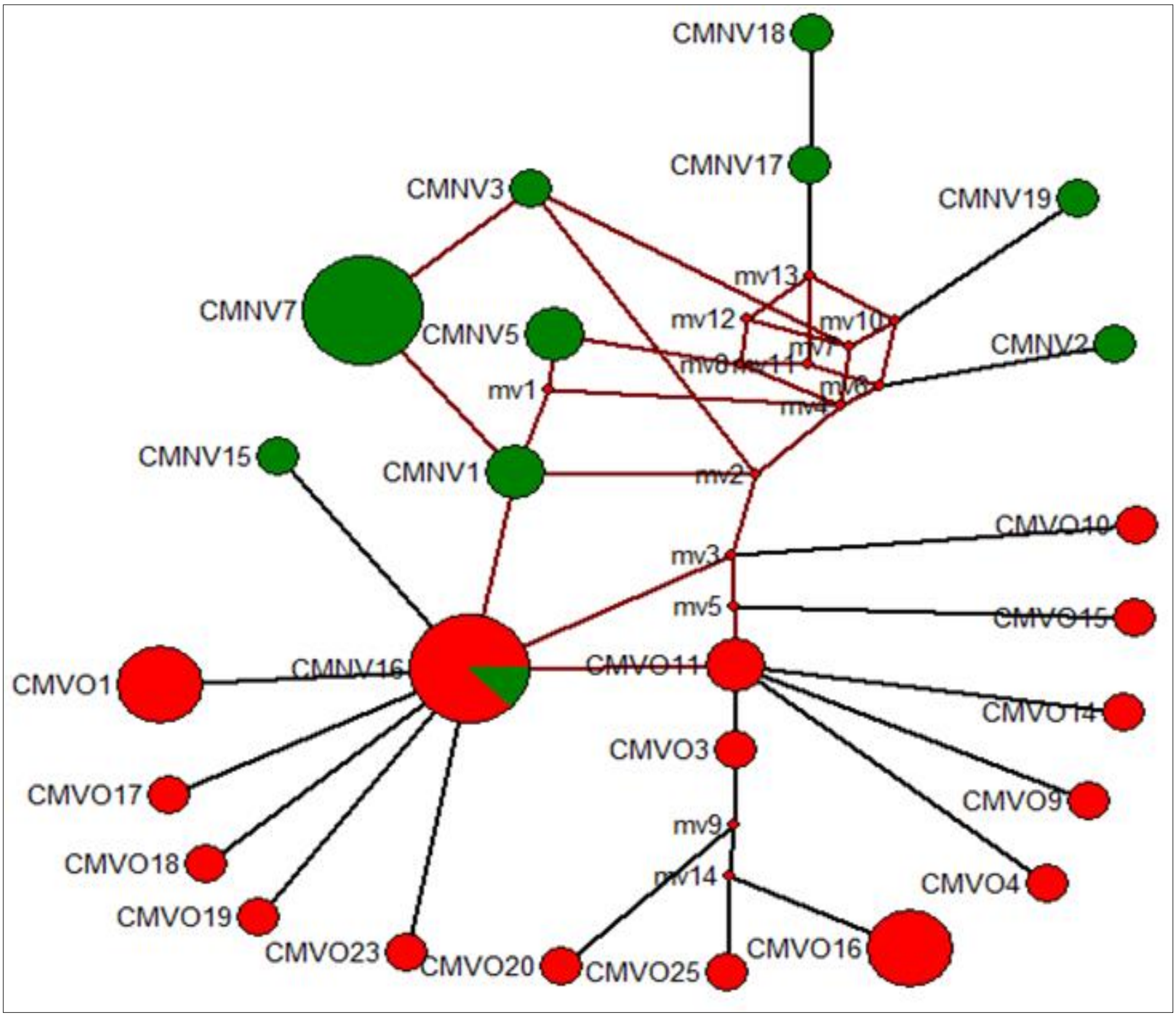
Table 7: SSD and Raggedness indices values

Parameters	Total population	Flight morph population	Flightless morph population
SSD p-value	0.0140 0.7500	0.0142 0.5900	0.0138 0.9000
Rag index (R) p-value	0.0274 0.6100	0.0184 0.9800	0.0364 0.2400

Phylogenetic relationships

1. Minimum haplotype network

Phylogenetic affinities of the inferred haplotypes with the sequence set reveal the presence of clustering of individuals into 25 haplotypes out of a total of 48 sequences. The topology of the haplotype network reveals a large number of individual haplotypes (approximately 18), of which 6 haplotypes are flightless morph and 12 haplotypes are flight morph, 5 intermediate haplotypes and 2 central major haplotypes grouping 8 individuals, of which H5 (CmNV7) is composed of flightless morph individuals and H7 (CmNV16) is composed of flight morph individuals. The connections between the central haplotype and the less frequent haplotypes vary from one to eight mutational steps (Figure 2). The frequency and proportion of nodes are related to the mutation rate and the median vectors indicate the number of mutational steps that favoured the differentiation of individuals from the central haplotype H5. The resulting network is characterised by a star shape with all haplotypes of the flight morph population deriving from a central haplotype H7 (CmNV16) of the flightless morphotype. The other central haplotype H5 (CmNV7) located two mutational steps away from H7 comprises only haplotypes of the flightless morphotype and is thought to be the origin of most of the other haplotypes of the flightless morphotype after 2 to 4 mutational steps. (Figure 2).



Legend: 🔴flight morphotype Flightless 🟢 morphotype

Fig 2: Haplotype network of the global Callosobruchus maculatus population according to the two morphotype

2. Phylogenetic reconstruction

The different phylogenetic reconstruction methods have given very similar topologies. The typology obtained with the Bayesian inference approach is much more resolved (Figure 3) and has been analysed. From the outer to the innernodes, a gradual and progressive differentiation of several individuals according to the two morphotypes is noticed. Overall there is a single large clade of the total population but containing a sub-clade of individuals from the flight morph population with a homogeneous clustering of individuals from each of the two populations studied and high bootstrap values ranging from 53 to 100%. The level of separation isolating the flight morphotypes from the flightless morphotypes is well defined by the Bayesian method with 62% bootstrap.

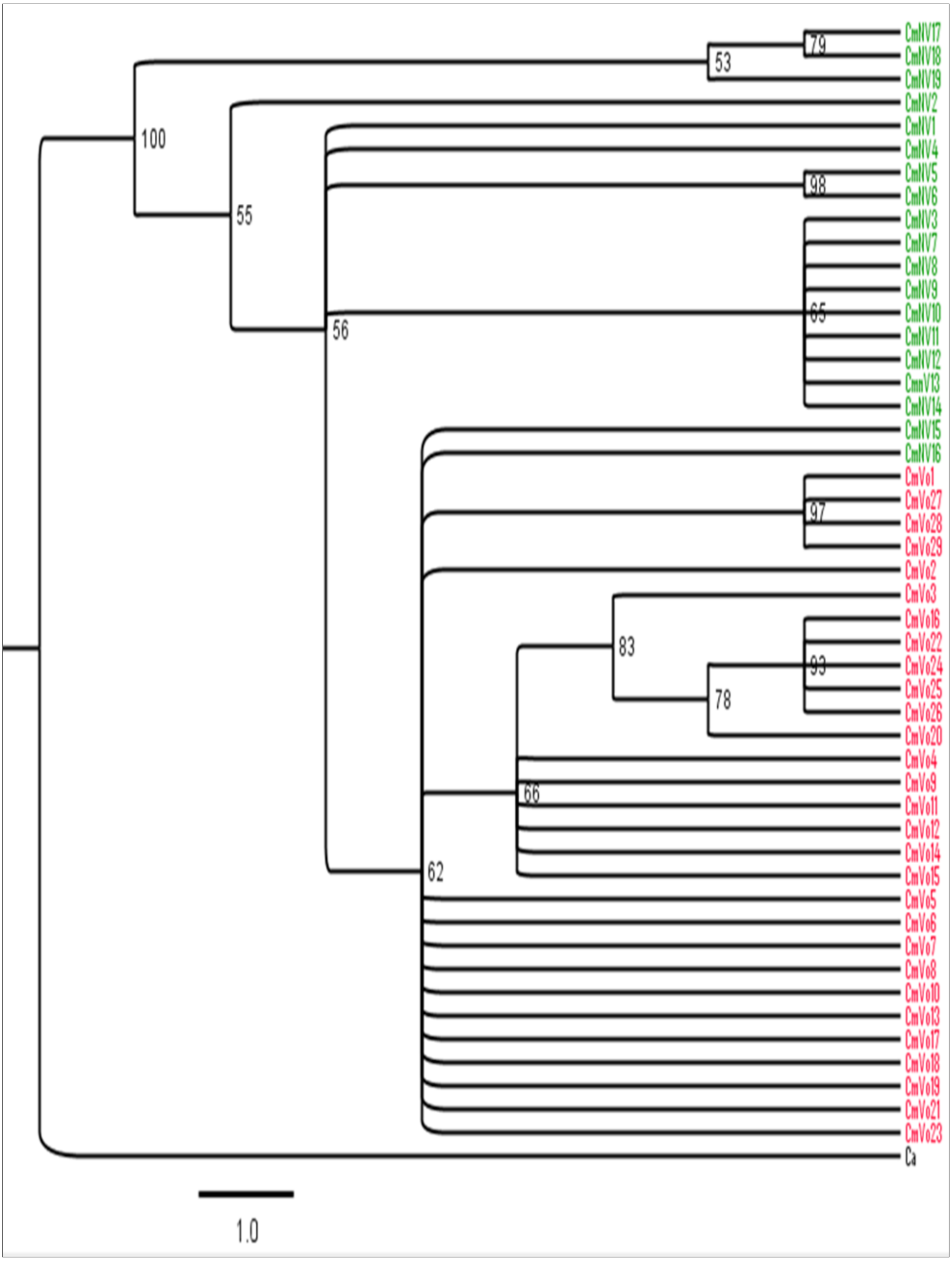


Fig 3: Phylogenetic reconstruction using the Bayesian method

Discussion

In the total population of Callosobruchus maculatus, we find a low variability and genetic polymorphism since most mutations are transitions (65.63%) which do not very often lead to changes in aminoacids and protein structure. However, when considering morphotypes, thereis a considerable decrease in genetic variability for flightless morphotypes subjected to transition mutations than for flight morphotypes subjected to transversion mutations which often induce a change of aminoacids and protein structure. Furthermore, the mutation rate R, relatively very high, suggests a weak differentiation of the flightless morphotypes compared to the flight morphotypes. Indeed, for all three populations, the low nucleotide diversity Pi and the high haplotypic diversity Hd reflect rapid population growth from an ancestral population with low effective size for a sufficient time for a recovery of haplotypic diversity (Hd) by mutation, but too short for the accumulation of strong differences between DNA sequences. These numerous mutations in the flightless morph population would probably be related to environmental or food pressures leading to the emergence of flight morph individuals. This also suggests an important of gene flow in the environment and the absence of reproductive isolation, so that individuals from different populations can reproduce without any genetic barrier. We can therefore conclude that there is a significant gene flow within the flightless morph population, possessing mainly genetic predispositions to mutations as soon as environmental conditions are met. Thus, there is a weak to moderate differentiation between the two populations or morphotypes. This suggests a negative correlation between geographic distance and genetic distance due to the fact that individuals from both populations live together in the same localities. The analysis of molecular variance confirms this differentiation as the AMOVA shows a significantly high percentage of variance suggesting the beginning of structuring between the two morphotypes. Moreover, the genetic tests seem to reveal that only the total population is expanding and this result is confirmed by the measurement of the distribution of genetic distances between individuals of a population taken in pairs with the mismatch graphs, which reveal a multimodal distribution reflecting a recent rapid growth of the three different populations. However, the strength of the mismatch distribution is supported by the value of the SSD and Raggedness indices which are all positive and insignificant. Considering the minimum haplotype network, there are 25 haplotypes or alleles out of the 48 sequences constituting the total population. However, two major haplotypes were found, one in the flightless morph population of 8 flightless morph individuals and the other in the flight morph population of 7 flight morph individuals and 1 flightless morph individual. All other majority haplotypes with 4 individuals are found in the flight morph population. This genetic diversity between the two populations is illustrated in the haplotypic network with a clustering of all the flightless morph alleles on one side (top and green) and the flight morph alleles on the otherside (bottom and red) but they are derived from a flightless morph ancestral allele. This suggests that some flightless morph individuals have genetic predispositions to spontaneously become flight morph individuals if environmental conditions allow it. The Bayesian tree analysis allows us to find this same evolutionary trend in the differentiation of the two morphotypes (flight and sedentary or flightless). This would be in agreement with previous hypotheses advocating the emergence of flight morph individuals within the flightless morph population on the one hand, and on the other hand, there is the presence of heterozygous flightless morph individuals with genetic predispositions in the form of unexpressed silent genes and whose genetic expression depends in part on environmental factors, allowing them to become flight morph individuals spontaneously. This favours the emergence of a new flight morph population formed by flight morph individuals leaving the fields and crops to fly and attack the granaries in stock as a result of punctual mutations responsible for new phenotypes favouring the ability to fly. In this same evolutionary trend, the Bayesian tree is much more resolved to show the emergence of the flight morph population from the flightless morph population. However, it appears two flightless morph individuals mixed and well embedded in the flight morph population (CmNV15 and CmNV16). This could be justified either by sampling or labelling error, or by an important gene flow linked to the absence of reproductive isolation between individuals of the two populations (panmixia). Individuals of this species first attack in the field through the female who lays her eggs on the still green fruits. When the eggs hatch, the larvae will enter the cowpea seeds and develop there. There may be several larvae in the same seed where development takes place to tally and entirely inside the seed, feeding on the nutrient reserves accumulated in the cotyledons. According to Ouédraogo [11], the differentiation of the flight morph from the initial flightless morph population is partly linked to environmental and demographic factors. This would trigger interactions between complex genes. Indeed, the creation of a phenotype is ensured by several biochemical reactions catalysed by different enzymes. Each enzyme is coded by a gene. It therefore often happens that the same characteris under the control of several genes: this is polygeny. Furthermore, epistasisis a form of interaction between non-allelic genes and one gene influences the phenotypic expression of another gene. In a population, individuals show a wide variety of phenotypes, which reflects a high genetic diversity of individuals. Indeed, natural selection is the pressure that the environment exerts on the individuals of a population, favouring the survival of the best adapted individuals and eliminating those with unfavourable alleles and traits. Natural selection is therefore the result of a struggle for survival leading to the successful development of fit individuals and the elimination of those considered 'unworthy'; it is as if nature hates the weak. In the end, genetic polymorphism provides some advantages to heterozygous individuals for certain traits and allows a better adaptation of the individual carrying the favourable allele, which increases the chances of survival of the species in the face of changing environmental conditions. Everything suggests that heterozygous individuals from the initial flightless morph infesting fields would be the future individuals of the flight morph infesting granaries by mutation and genetic differentiation under the influence of environmental pressure and natural selection for expression of silent or recessive allèles. In this work [15], Sano demonstrated that the imaginal polymorphism of C. maculatus results from an interaction between environmental and genetic factors. In addition, there would be a maternal effect, eggs laid at the end of the spawning period produce more flight morph species than those laid at the beginning of the spawning phase.

Conclusion

Ecological pest control, respectful of consumers health and the environment, not relying on alternatives to chemicals, can be developed with the knowledge and control of the genome of pest populations such as Callosobruchus maculatus responsible for the primary infestation of cowpea or Vigna unguiculata. It is imperative to reduce the attacks of this pest on foodstuffs by decreasing the rate of emergence on the one hand and by interrupting the chain of infestation from the crops to the stored cowpea granaries on the other hand by identifying the gene(s) responsible for the expression of the flight morph phenotype. If the enzymes required for the flight morph phenotype can be prevented, the alleles involved could be modified by exposing all flightless morph individuals to mutagenic factors. With the development of genomics, proteomics, molecular and cell biology on the one hand and classical genetics on the other, it would be possible to determine the proteins differentiating the flight morph in order to identify the genes involved on which humans could intervene effectively to reduce the emergence of flight morph and losses. Indeed, the results of analysis with Cyt B clearly revealed a moderate differentiation between the two populations (two morphotypes) inducing a weak structuring leading to sympatric speciation. Furthermore, exposure of crops and stored food stuffs to certain physical conditions (UV rays, well-dosed X-rays or electromagnetic waves) could be a mutagenic factor for an alteration of these targeted genes, thus preventing a strong emergence of flight morph individuals of Callosobruchus maculatus from the sedentary flightless morph population. This would then contribute to the control of these insect pests, while at the same time helping to reduce post-harvest cowpea losses for some farmers, thus contributing significantly to food self-sufficiency.

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