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Genetic structuration of *Rastrococcus invadens* populations in Senegal (Casamance and Thies)

Amadou Fall, Fawrou Seye, Tofféne Diome, Mamour Toure, Raymond Demba Ndiome and Mady Ndiaye

Abstract

Rastrococcus invadens (Homoptera, Pseudococcidae) or cochineal is an insect pest of mango trees and several other fruit trees including citrus. It belongs to the branch of Arthropods, class of insects, to the order of orthoptera, to the super family of Coccoidea and to the family Pseudococcidae. The cochineal is native to Southeast Asia and was first identified in Dakar (Senegal) in 1995. Since then it has been widely used throughout the country and more particularly in the two most fruit-producing regions: Casamance and Thies. To study the genetic structure of the populations of *Rastrococcus invadens*, we have chosen the three species of host plants most attacked, namely the mango tree, the lemon tree and the orange tree. On each foot, we randomly harvested 10 insects. This allows us to have a total of 60 individuals, including 30 in the Casamance natural region and 30 in the Thies region. Samples are coded according to the area and type of plant in which they were collected using the first letter of the genus name, the first letter of the region of origin and the first letter of the host plant. The same individuals stored in 70% alcohol were used for extractions, PCR (Chain Reaction Polymerase) and sequencing. The analysis is done by several software in order to output the different parameters of the study. The results obtained in genetic diversity show six groups of individuals according to host plants and study areas which confirm those of morphometry. However, since these groups have very small genetic distances, they constitute a single species of *Rastrococcus invadens*.

Keywords: *Rastrococcus invadens*, host plants, study area, genetic diversity, morphometric characterization, pest

1. Introduction

Scale insects (Hemiptera: Pseudococcidae) are a large family of insects with around 2,000 species described in more than 270 genera [1]. The family Pseudococcidae has a worldwide distribution, but it is more common in the subtropical and tropical regions [2]. Their name derives from a white, waxy secretion found on the bodies of adult females and nymphs of most species. Many members of this family are pests of a wide variety of crops in tropical, subtropical and temperate regions. Certain species cause a very significant negative impact on the yield and quality of production. Losses in economic crops due to large populations of mealybugs and excessive production of honeydew which serves as a substrate for mold growth [3] or the transmission of viruses which can radically reduce crop yields [4]. The taxonomy of mealybugs has generally been based on the morphology of the characters of adult females [5], and on few studies that have focused on adult males. This is likely due to the ephemeral nature of adult males and the difficulty in capturing them. In addition, some mealybug species are parthenogenic [5], thus eliminating the possibility of using males to identify these species and create inconsistencies in phylogenetic studies. Because of these factors, the use of adult females remains the standard for taxonomy, phylogenetic analysis and description of species in the Pseudococcidae family. Recently, advances in genetics have made DNA barcodes a practical means of classifying individuals of Pseudococcidae and comparing them morphologically. Several studies on the molecular variation of individuals of Pseudococcidae have been made [6]. But it is only recently that phylogenetic studies have been integrated morphological data with DNA sequences [7]. Faced with this numerous damages, several control techniques have been developed, going from chemical struggles with their consequences on the environment, to physical struggles (felling and incineration of trees) which constitute a real shortfall for farmers. Therefore, a better knowledge of the bio-ecology of the insect and its genetic structure in Senegal in the two most fruit-producing areas (Casamance and Thies) is necessary to be able to adopt an adequate control.

The objective of this study is therefore to determine the genetic diversity of the different populations of *Rastrococcus invadens* and their structures according to the agro-ecological zones and the host plants most affected.

2. Materials and methods

2.1 Sampling

The study areas were chosen according to the importance of fruit production and the degree of infestation. Sampling was carried out at two sites in Senegal, namely Diatock in the Casamance natural region and Khay in the Thies region.

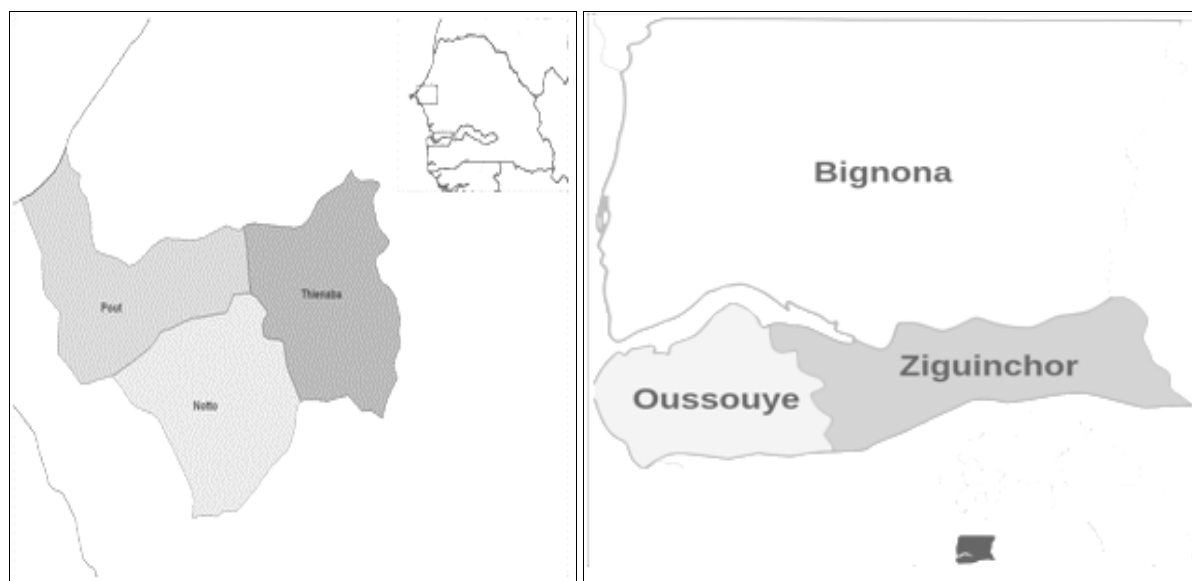


Fig 1: Agro-ecological zones sampled

In our sampling strategy, we chose the three most attacked host plant species, namely the mango, lemon and orange trees. On each foot, we randomly harvested 10 insects. This allows us to have a total of 60 individuals, including 30 in the Casamance natural region and 30 in the Thies region. Samples were coded according to the area and type of plant in which

they were collected using the first letter of the genus name, the first letter of the region of origin and the first letter of the host plant. The data is described in table 1 below. Insect pests (*R. invadens*) were then collected in the laboratory and stored in 96% alcohol for the PCR-Sequencing technique.

Table 1: Summary table of the sampling

Study area	Locations	Host plants	Number of individuals	Codes
Casamance	Diatock	Mango	10	RCM
		Lemon	10	RCC
		Orange	10	RCO
Thies	Khay	Mango	10	RTM
		Lemon	10	RTC
		Orange	10	RTO

2.2 Genetic study

2.2.1 DNA extraction

The Qiagen DNeasy Tissue kit method was used for DNA extraction from insects. For this, the insect was dissected, only the thorax, the head and the legs were used for the extraction by placing them in a 1.5 ml tube in which 180 μ l of ATL buffer were added for dissociation of the tissues and an individualization of the cells then 20 μ l of proteinase K to degrade all the proteins after an incubation at 55 ° C. for 3 hours overnight.

2.2.2 Cytochrome b PCR

It was based on selective *in vitro* amplification of a particular template DNA sequence by extension of two primers by a DNA polymerase, in the presence of deoxyribonucleotides (dNTP) and Mg²⁺ ions. The amplification was carried out in a reaction volume of 25 μ l containing 18.525 μ l of ultrapure water, 2.5 μ l of non-colored buffer (10x), 1 μ l of MgCl₂ (25mM), 0.175 μ l of each primer (100 μ M), 0.125 μ l Taq (5U / μ l) and 2 μ l DNA extract.

2.2.3 Cytochrome b sequencing

Sequencing was a technique that involves determining the nucleotide succession of a DNA fragment. It was produced in South Korea. Thus, it makes it possible to highlight point mutations, by comparing the sequences of the same gene in different individuals. This is done thanks to a specific PCR reaction, using in addition to the usual compounds (DNA-matrix, polymerase, primers, dNTPs, Mg²⁺), modified nucleotides, such as dideoxynucleotides (ddNTPs).

2.3 Genetic analyzes

The sequences were analyzed by several software programs in order to extract several parameters from the study. Thus, the sequences were aligned, checked and corrected by BioEdit software version 7.0.5.3 2005. To determine the number of haplotypes, conserved, variable and informative sites sparingly, it is the software dnasp.5.10.01 from Rozas *et al.*, 2010^[9] which has been used. Haplotypes designate a group of individuals with the same sequence. To this was added the use of Harlequin software version 3.1 from Excoffier *et al.*, 2005

[10] to be able to study genetic differentiation, molecular variance within populations and between populations. For the implementation of haplotype networks, the sequences were first transformed by dnasp.5.10.1 from Rozas *et al.*, 2010 [9] in phylip format then analyzed by TCS1.21 from Clément *et al.*, 2005 [11] in order to visualize the number of mutational steps. The parameters of demographic evolution of populations such as the D of Tajima and the FS of Fu are determined by harlequin version 3.1 of Excoffier *et al.*, 2005 [10]. The phylogenetic reconstructions of the trees by the Neighbor-Joining method, the maximum parsimony and the maximum likelihood, were implemented by the MEGA5.05 software from Tamura *et al.*, 2011 [12]. However, the elaboration of trees by the Bayesian inference method was carried out by Mrbaye software version 3.1.2 of Huelsenbeck and Ronquist, 2001 [13] by first transforming the sequences into phylip format.

3. Results

3.1 Polymorphism and genetic diversity

3.1.1 Polymorphism

The H1 haplotype contains 14 individuals, H2: 10 individuals, H3: 12 individuals, H4 and H5 each contain 16 individuals and H6: 11 individuals.

3.1.2 Haplotypic and nucleotide diversity of *R. invadens* populations in agro-ecological zones

We can clearly see that the nucleotide diversity is zero (0) in the different agro-ecological zones studied and according to the plants, with the exception of the Casamance mango tree (RMC: 0.15385) and the lemon tree of Thies (0.11765) (table 2).

Table 2: Genetic diversity of *R. invadens* populations in the study areas

	RCC	ROC	RMC	RCT	ROT	RMT
N	13	10	13	17	11	15
H	1	1	2	2	1	1
S	0	0	3	15	0	0
Hd	0	0	0.15385	0.11765	0	0
Pi	0	0	0.00062	0.00235	0	0

N: number of individuals, H: number of haplotypes, Hd: haplotypic diversity Pi: nucleotide diversity, S: segregation

Table 5: The indices of neutrality of the populations of *R. invadens* encountered in different areas

	RCC	ROC	RMC	RCT	ROT	RMT
DT	0	0	-1.65231	-2.31940	0	0
P-value	1	1	0.02700	0	1	1
Fs	0	0	0.97596	4.54061	0	0
P-value	N.A.	N.A.	0.59800	0.96100	N.A.	N.A.

The irregularity index (rg) is significant only in populations from the Casamance mango tree (0.76331) and the Thies lemon tree (0.80623). However, it is zero for all other populations.

This same result is observed on all the other parameters with zero P-value for all populations except those from the mango tree from Casamance (0.1200) and the lemon tree from Thies (0.0900). The SSD value is also zero for all populations except that from the Casamance mango tree (0.03430) and the Thies lemon tree (0.02032) (Table 6).

site

RCC: *Rastrococcus* from the Casamance lemon tree, ROC: *Rastrococcus* from the Casamance orange tree, RMC: *Rastrococcus* from the Casamance mango tree, RCT: *Rastrococcus* from the Thies lemon tree, ROT: *Rastrococcus* from the Thies orange tree, RMT: *Rastrococcus* from the Thies mango tree.

3.1.3 Genetic differentiation of *R. invadens* populations between study areas and host plants

It is noted that the values of Fst obtained between the different populations studied are everywhere strong with, however, values substantially equal to 1 (Table 3).

Table 3: Genetic differentiation (Fst of *R. invadens* populations between study areas and host plants

Fs de Fu	D de Tajima	R2
0.37613	-0.05157	0.09578
12.54340	12.48809	8.26505

3.1.4 Demographic evolution of *R. invadens* populations in the two areas studied

Considering the total population, we obtain a negative Tajima D (-0.05157) and a positive Fs (0.37133) but are not significant. The value of R2 (0.09578) is also not significant and the values of p-values almost everywhere equal to 1 with the exception of individuals from the Casamance and Thies mango trees where the value is substantially zero (Table 4).

Table 4: Demographic parameters of the total population of *R. invadens*

	RCC	ROC	RMC	RCT	ROT	RMT
RCC	0					
ROC	1	0				
RMC	1.000	0.967	0			
RCT	0.917	0.907	0.911	0		
ROT	1	1	0.972	0.902	0	
RMT	1	1	0.969	0.933	1	0

The values of D of Tajima (DT) are not significant in each agro-ecological zone, the p-value (P) are all greater than 0.05 and those of Fu (Fs) significant in populations from the Casamance mango tree (0.97596) and very significant in populations from the Thies lemon tree (4.54061) (table 5).

Table 6: Demographic parameters for each population of *R. invadens*

	RCC	ROC	RMC	RCT	ROT	RMT
R2	0.16419	0.16241	0.16254	0.16125	0.16191	0.16252
P-value	0.5	0.507	0.523	0.518	0.513	0.499
SSD	0	0	0.03430	0.02032	0	0
P-value	0	0	0.12000	0.09000	0	0
rg	0	0	0.76331	0.80623	0	0
P-value	0	0	0.64000	0.77000	0	0

The “mismatch distribution” curves where the distribution of the number of differences between the haplotypes taken two

by two are multimodal (Figure 2).

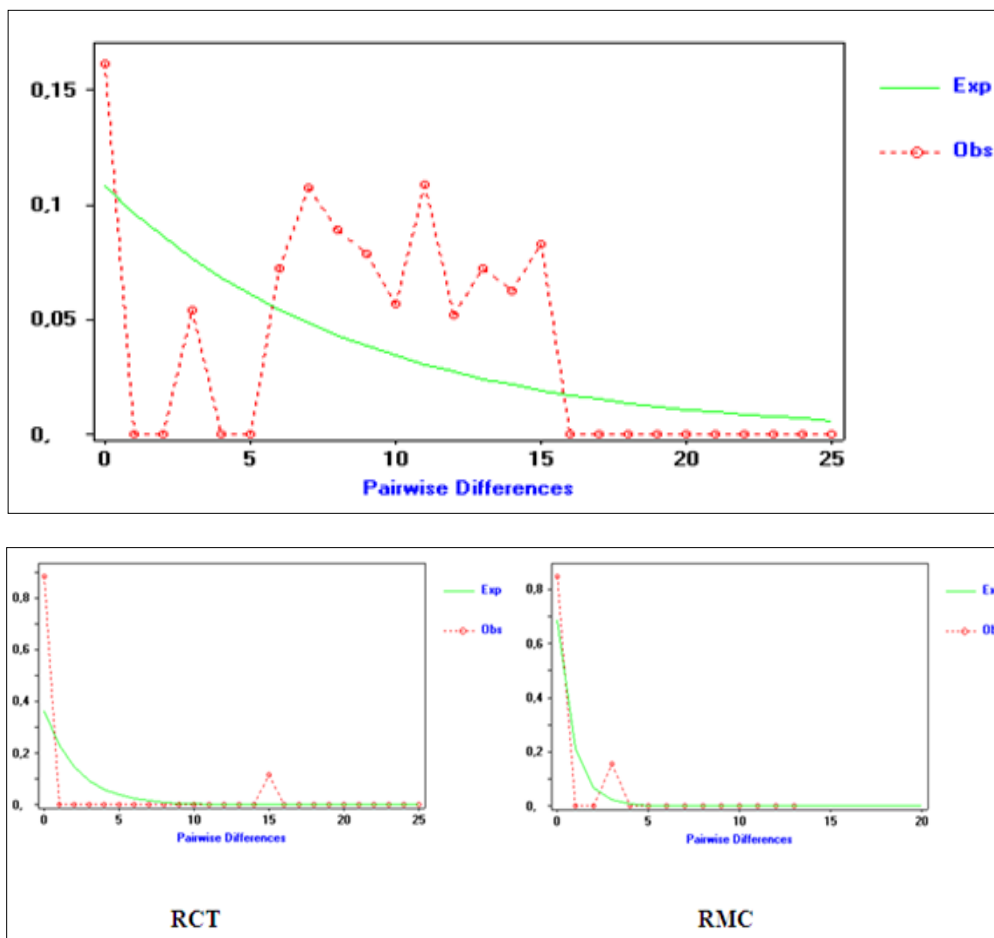


Fig 2: Distribution of the number of differences between haplotypes taken two by two (mismatch distribution) for the populations of *R. invadens* subservient in the different agro-ecological zones

3.2 Genetic distance of *R. invadens* populations between study areas

The genetic differentiation between the populations of the

Casamance zone and those of This on the one hand and that between the host plants on the other hand is weak and not significant (table 7).

Table 7: Genetic distance between populations

Species 1	Species 1	Dist	Std. Err
RCC-	ROC-	0.009	0.003
RCC-	RMC-	0.003	0.002
ROC-	RMC-	0.010	0.003
RCC-	ROT-	0.011	0.003
ROC-	ROT-	0.012	0.004
RMC-	ROT-	0.012	0.004
RCC-	RMT-	0.008	0.003
ROC-	RMT-	0.015	0.004
RMC-	RMT-	0.009	0.003
ROT-	RMT-	0.013	0.004

3.2.1 Phylogenetic haplotype networks and reconstructions

3.2.1.1 Haplotype network and distribution of *R. invadens* haplotypes

Haplotype networks allow us to see the genetic differences and the links between different haplotypes. Each disc corresponds to a haplotype, and their size is proportional to the number of individuals corresponding to the haplotype. The white circle separates two mutational steps between haplotypes. The majority haplotype (16 individuals) is found in the population from the Thies mango tree. Likewise, the

average haplotype (13 individuals) is found in the population from the Thies lemon tree. In the first, it is the population from the Thies lemon tree which is more represented and in the second haplotype, it is the population from the Casamance lemon tree which is the majority. However, there are neither individual haplotypes nor private haplotypes. Six (6) haplotypes were encountered in the two agro-ecological zones studied (H1, H2, H3, H4, H5 and H6) with three haplotypes in Thies (one on mango tree, one on lemon tree and one on orange tree) and three in the Natural Casamance (one on mango tree, one on lemon tree and one on orange tree).

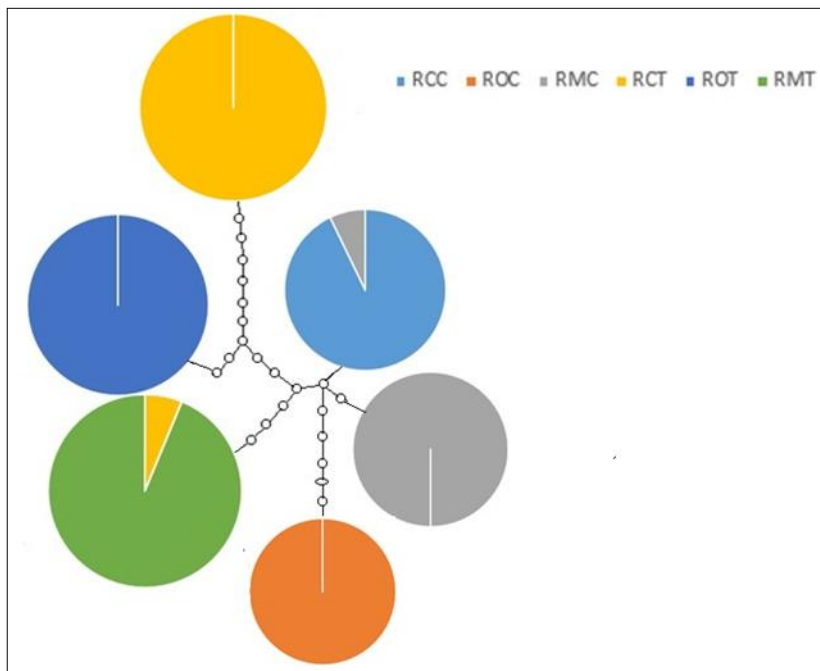


Fig 3: Cyt b haplotype network of *R. invadens* populations encountered in the study areas

3.2.1.2 Phylogenetic reconstructions

The phylogenetic trees were constructed by the Neighbor Joining method, maximum parsimony, maximum likelihood and by the Bayesian inference method. The phylogenetic trees of the *R. invadens* populations were rooted by an individual of a *Bactrocera* species. The most resolving trees were obtained by the Bayesian inference method. In *R. invadens*, the phylogenetic tree of Cyt individuals, b presents six very distinct groups. However in different trees, the result is almost the same.

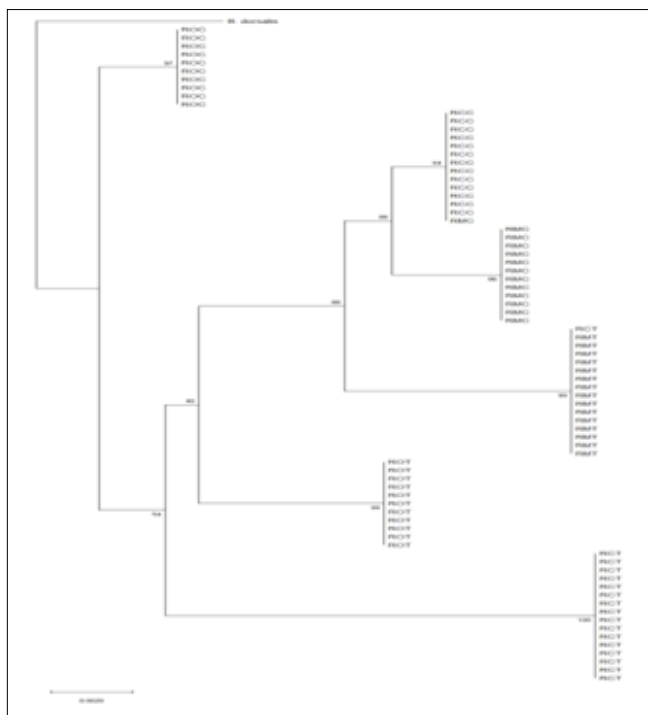


Fig 4: Phylogram of individuals of *R. invadens* from Senegal using the Neighbor-Joining method

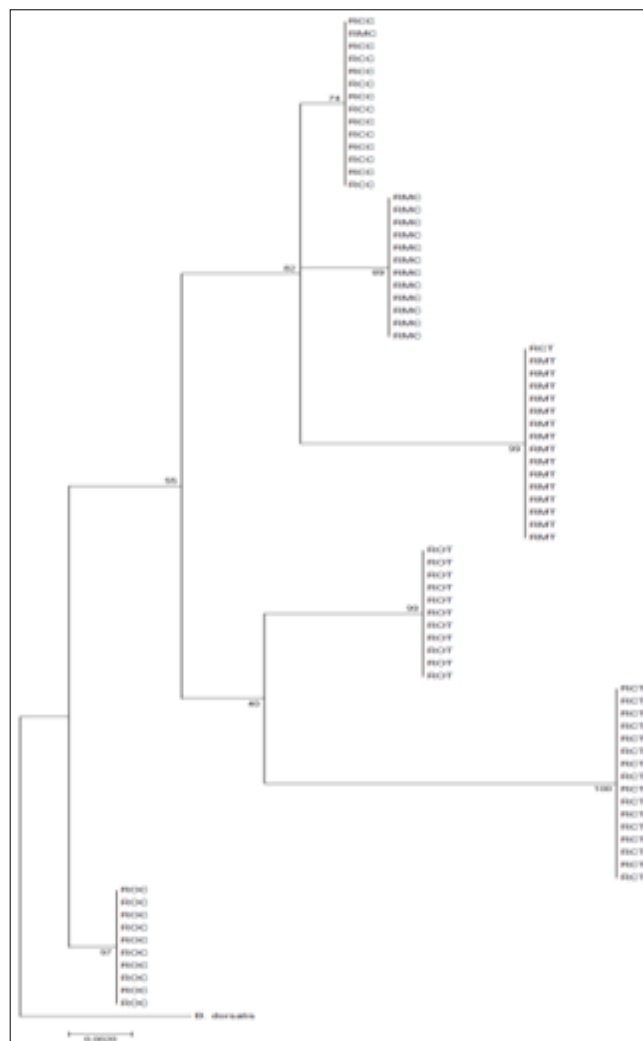


Fig 5: Tree of maximum parsimony of *R. invadens* individuals

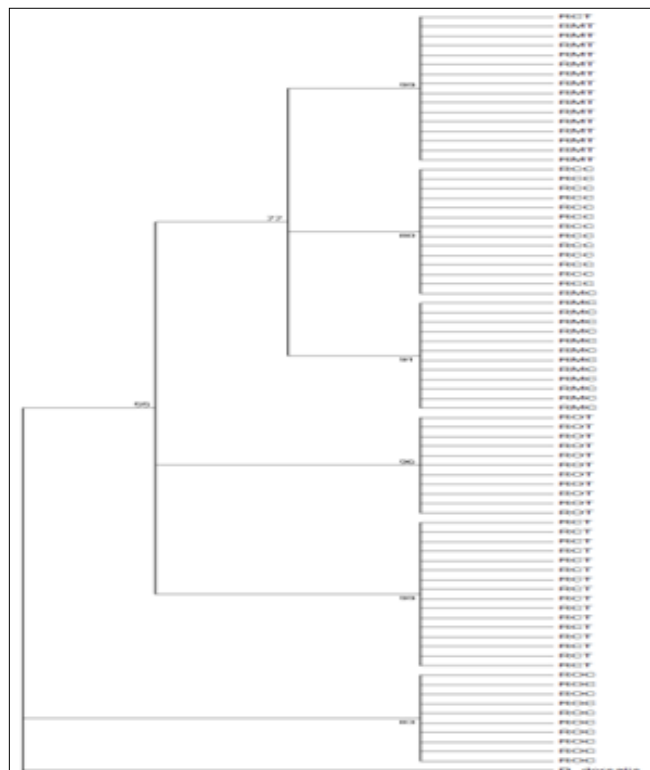


Fig 6: Maximum likelihood tree of *R. invadens* individuals

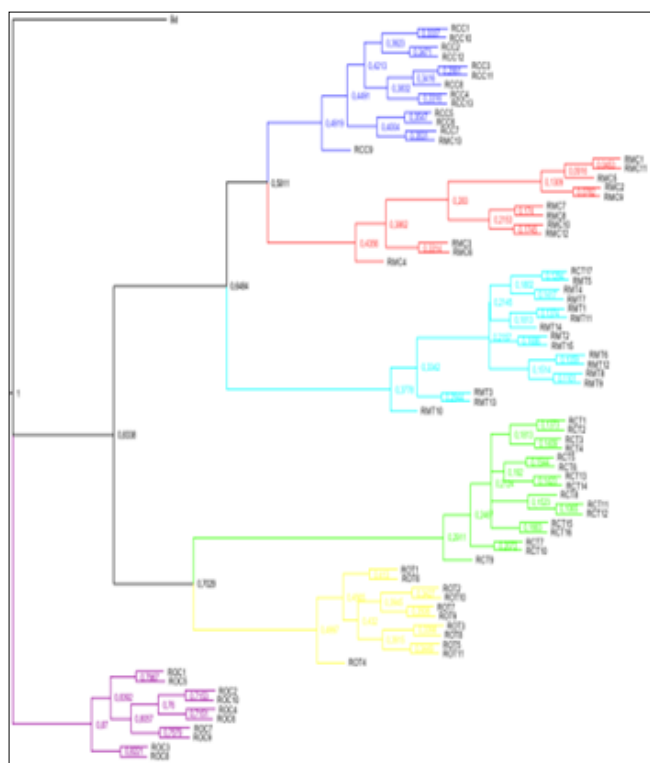


Fig 7: Bayesian tree of *R. invadens* populations using the Bayesian inference method

4. Discussion

The objective of this study is to determine the diversity and genetic structure of *R. invadens* according to the study areas (Thies and natural region of Casamance) and host plants. This study is necessary for a better fight because the mealybugs (Hemiptera: Pseudococcidae) are real very polyphagous pests. The results obtained with the mitochondrial cytochrome b gene show that the populations of *R. invadens* encountered in Senegal contain different haplotypes. At least 6 haplotypes of

R. invadens circulate in the different host plants and the agro-ecological zones sampled. In agro-ecological zones, the strong haplotypic and nucleotide diversities observed would testify either to the stability of these populations with large effective size, or to an admixture of both. The same assumptions are noted for the population of host plants. A strong genetic diversity is noted in Thies. This could lead us to consider that Thies would be the home of the infestation because the insect was observed for the first time in Senegal in the Niayes area [14]. The difference that exists for the evolution of these populations at the molecular level could be explained by the variable nature of Cyt. b. According to a report by GERAD, 2006 [15], in most cases the insect can be transported on plant material, by travelers ... Indeed, in all the localities studied, mango and citrus fruits have become commercial crops. This could lead to a large gene flow by the introduction of new individuals from border areas and a rapid multiplication of the species by the availability of food throughout the year. Indeed, there is a significant human displacement in Senegal thus facilitating the transport of plant material (fruits, vegetables from one zone to another). The widening of the study of the genetic structuring of the different populations of *R. invadens*, shows at the level of Cyt. b high haplotypic and nucleotide diversity, resulting in a large genetic flow between populations. Since the mango and citrus fruits do not have the same period of fruit production and the insect was first discovered on mango [16], it can be considered that citrus and other host plants are refusal plants for *R. invadens*. The study of genetic differentiation revealed that between the populations of *R. invadens* from Thies and Casamance, there is little genetic differentiation. This genetic differentiation is also noted between populations from different host plants. This observed genetic differentiation can be considered as the genetic differentiation that exists between the different localities, because each is encountered in a specific agro-ecological zone and according to the host plants which do not have the same organoleptic taste. Therefore, we can assume that there is a structure of *R. invadens* within these different localities, as in the case of *Tribolium castaneum* [17]. The haplotype study shows that *R. invadens* has 6 haplotypes in all of the study areas, with H4 and H5 as the majority haplotypes (16 individuals each). The establishment of the *R. invadens* haplotype network shows that some haplotypes have the H4 haplotype as their common ancestor. The latter is not only the majority haplotype, but can be considered to be regional, as it is present in Thies and Casamance. In this network, the link between the H4 and H6 haplotype on the one hand and H1 and H3 on the other hand is very remarkable. In H2, we only find populations from the Casamance orange tree and in H5 we only find populations from the Thiès orange tree. The particularity of the Cyt haplotype network. b is that haplotypes H1 (RCC), H2 (ROC) and H3 (RMC), all of Casamance origin, would come from haplotypes H4 (RCT), H5 (ROT) and H6 (RMT) which come from Thies. Consequently the individuals of Casamance would come from individuals of Thies which could be considered as the ancestral haplotype. Based on the values of D from Tajima and FS from Fu du Cyt. b, the populations of *R. invadens* from Thiès and from Casamance show excesses of rare variant and are the target of natural selection favoring genetic diversity. By considering each host plant and each agro-ecological zone, we see that the value of D of Tajima is zero or even negative; which probably indicates a random evolution of the populations of *R. invadens* in these different

host plants and agro-ecological zones. Indeed, according to Excoffier, 2005^[10], a negative Tajima D could correspond to a demographic expansion. In the populations from the Casamance mango tree from the Thies lemon tree, the values of D from Tajima (RMC: -1.65231; RCT: -2.31940), (-0.00864) are negative but not significant. This shows that these populations are in demographic balance. The multimodal curves observed would indicate stable populations across the entire Cyt gene. b. This stability would be due to an adaptation of *R. invadens* to their environment by the presence perhaps of endocytobionts which according to Hubert C., 1997^[18] plays an important role in the accommodation of the host in relation to the variations of the environment. In general, the populations of *R. invadens* are almost genetically identical and stable. This is confirmed by the table 7 and the mismatch distribution curves at the level of the Cyt gene. b. *R. invadens* would present a structure at the level of certain agro-ecological zones. However, the development of the tree of individuals of *R. invadens* shows that the grouping is done not only according to the zones but also according to the host plants.

5. Conclusion

At the end of this study, only one species of the genus *Invadens* was identified in the different agro-ecological zones. Its evolution is that of a stable population with large effective size. This stability is due to an adaptation of *R. invadens* to its environment. So new strategies to control these crop pests must be considered. In populations of the same species, the Cyt gene. b has different trends. The genetic differentiation between agro-ecological zones as well as between host plants is sometimes different at the level of the *R. invadens* gene where we have identified a large number of populations. From the analysis of the results, a structure of *R. invadens* between certain host plants is noted. According to the molecular variance and the correlation between geographic distance and genetic differentiation, *R. invadens* shows a genetic structure according to agro-ecological zones and host plants. On the other hand, the development of the haplotype tree presents groupings of haplotypes according to agro-ecological zones and host plants.

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