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Genetic structure of *Rastrococcus invadens* populations on citrus in Senegal

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Abstract

Citrus is a strategic sector in most producing countries, thus playing a significant socio-economic role. However, it is confronted with phytosanitary problems, including insect pests: the mealybug or *Rastrococcus invadens*. It causes direct and indirect damage to host plants (mango and citrus). For a better knowledge of the insect, a study based on the genetic structuring of the populations of *Rastrococcus invadens* on citrus fruits in Senegal was carried out. This study was carried out in four farms (Diatock and Oussouye) located in the natural Casamance region and (Santhie and Khay) in the Thiès region. In each farm, we chose ten individuals of *R. invadens* from lemon, orange, mandarin and grapefruit. The PCR-sequencing technique was used using the mitochondrial cytochrome b gene. The results showed six groups of *Rastrococcus invadens,* with five groups common to Orange, Grapefruit, Mandarin, Lemon and the other group specifically for Orange and Grapefruit. The value of Fst=0.286 (AMOVA) showed that this structuring is weak overall, unlike the breasts of the localities. The demo-genetic tests and the mismatch distribution curves showed that the populations of *Rastrococcus invadens* are globally stable.

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Introduction

Cultivated on a very large scale, citrus fruits are at the top of fruit production in the world. In 2012, world production exceeded 131 million tonnes (FAO, 2014). In Senegal, as in most West African countries, fruits constitute food products with high nutritional and commercial value (Dembele *et al.*, 2013). They contribute to the improvement of social well-being and the state of health of populations (FAO, 1999).

The annual production of fruits and vegetables is globally estimated at 800,000 tons, of which rarely 1% is exported (Niang, 2006). This production comes mainly from Casamance, Thies and rural Dakar. Market and financial value provide important currency to producers (Dembele *et al.*, 2013).

Nowadays, fruit production is threatened by phytosanitary problems, the most important of which are fruit flies (family Tephritidae) (Norrbom, 2004) and the mango mealy bug *Rastrococcus invadens* (Williams, 1986) (Homoptera: Pseudococcidae) (N'guetta, 1995). The latter pest was accidentally introduced into Africa in the early 1980s from South-east Asia, where it originated (Williams, 1986).

Rastrococcus invadens (Willams, 1986) (Homoptera, Pseudococcidae) or mealybug is an insect pest of mango trees and several other fruit trees including citrus. It is native to South-east Asia and was first identified in Senegal in Dakar in 1995 (Han *et al.*, 2007). Therefore, it is widely practiced throughout the country and, more particularly, in the two most fruit-producing areas: Casamance and Thies.

In Senegal, these mealybug populations are low from September to February and particularly during the harmattan. The highest numbers are observed from March to August, with a maximum between June and August (Hala *et al.*, 2004). This low mealybug population could be explained by the fact that, on the one hand, between August and September, most of the insects are washed away by the rains and on the other hand, the mealybugs are attacked by other predatory insects such as guards. Red crabs, crabs, or even parasitoids. The duration of development, fecundity and survival of *R. invadens* depend on abiotic (in particular temperature) and biotic (quality of the host plant and natural enemies) factors (ANSES, 2015). The spread of the mealybug seems to be ensured essentially by man through his activities: transport of plant material attacked (seedlings, grafts, leaves, wood) and working material (carts, clothes) (Hala *et al.*, 2004). The losses related to the damage of these insects are estimated throughout the world at several billion dollars, according to (Norrbom, 2004).

It causes direct damage by punctures which lead to the loss of sap and indirect damage by the production of honeydew and sooty mold, which form on the surface of leaves and fruits (Hala *et al.*, 2004).

However, Fall *et al.*, 2017 carried out an inventory of edible host plants and assessed the degree of infestation of *Rastrococcus invadens* (Willams, 1986) (Homoptera, Pseudococcidae) in Senegal. Matokot *et al.*, 1992 studied the population dynamics of *Rastrococcus invadens* (hom: Pseudococcidae) in Congo. Agricola *et al.*, 1989 carried out a controlled study of *Rastrococcus invadens* (Williams, 1986) (Hemiptera: Pseudococcidae) in Togo by the introduction of Gyranusoidea tebygi (Hymenoptera: Encyrtidae). A morphometric characterization of *Rastrococcus invadens* on citrus was made by Fall *et al.*, 2019.

Nevertheless, although various studies have been highlighted by many authors, enormous losses and damage have been observed at the level of host plants: mangoes and, in particular, citrus fruits. As a result, the question of the genetic structure of populations of *Rastrococcus invadens* dependent on citrus arises. The general objective of this study is to contribute to the knowledge of the genetic structure of populations of *Rastrococcus invadens* dependent on citrus in Senegal.

In order to study the structuring, our general objective will be broken down into two specific objectives:

First, study the genetic diversity of populations of *Rastrococcus invadens* dependent on citrus in Senegal, Second, characterize the genetic structure of *Rastrococcus invadens* populations on Grapefruit, Orange, Lemon and Mandarin in Senegal.

Material and methods

Study sites

In order to obtain information at the site level, surveys and surveys were carried out in the regions of Thies and Natural Casamance, which are the most productive areas of fruits in Senegal. Geographical coordinates, humidity and temperature are obtained using GPS, hygrometer and thermometer, respectively.

Thies region

The Thies region is one of the fourteen administrative regions of Senegal and covers an area of 6601 km². It is bounded to the East by the regions of Diourbel and Fatick, to the west by the region of Dakar and the Atlantic Ocean, to the north by the region of Louga and to the south by the region of Fatick. The Thies region has the following geographical coordinates: latitude 14° 45' 43 North and longitude 17° 17' 57 West.

This region has two coastlines, one to the north with the Grande Côte sheltering the Niayes market gardening area and the other to the south with the Petite-Côte, which is one of the most tourist areas of Senegal. We chose the Niayes, more particularly the commune of Pout, the second most productive area of fruit after Casamance. The climate of the region is influenced by sea currents because the region is located in a transition zone subject to the influence of the maritime trade winds and the harmattan with an average temperature of 32°C. The rainfall varies between 300 to 600 mm from East to west.

The plant formations are dominated by Elaeis guinensis, *Cocos nucifera* and aquaphilous species (Nymphea lotus, Phragmites vulgaris, etc.) on the lowlands with very humus-rich soils; by Parinari macrophylla, Acacia alba, Acacia seyal, Balanites aegyptiaca, with Euphorbiaceae, Combretaceae and grasses such as Cenchrus biflorus, Andropogon sp., Eragrostis sp., on the Gogolian red dunes; by Opuntia tuna, Maytenus senegalensis on yellow and white dunes. In addition to this natural vegetation, there are protective plantations of Casuarina equisetifolia.

Natural Casamance region

Casamance is a part of Senegal that covers a total of 52,000 km², whose only relief is represented by the foothills of Fouta Djalon in the south-east of the territory. It is bordered to the East by Mali, to the west by the Atlantic Ocean, to the north by Gambia and to the south by Guinea-Bissau and Guinea Conakry.

In this region, we sampled at the level of the departments of Bignona and Oussouye, which constitute the most productive zones of fruits in Senegal. In the department of Bignona our sampling was carried out in the locality of Diatock: latitude 14° 45' 43 North, longitude 17° 17' 57 West and altitude of 17 m from the sea. In the department of Oussouye, it is the locality of Loudia wolof which was chosen: latitude of 12° 39' 59 North of longitude 16° 15' 26 West and altitude of 40 m from the sea. The climate of the region is of the Sudano-Guinean type, characterized by a period wet from June to October (summer) but here called the rainy season or wintering. The rainfall varies between 800 to 2000 mm from East to west. The temperature is substantially equal in the different areas studied and is around 30°C (29.8°C in Bignona and 30.1°C in Oussouye).

In Lower Casamance, the vegetation is dense semi-dry forest type for the most part. It is characterized by sub-Guinean species, the most representative of which are Khaya senegalensis (Cailcedrat), Afzelia africana (Linke), Parinari excelsa (Mampato), Ceiba pentandra (Fromager), Chlorophora regia (Iroko), Antiaris africana (Tomboiro), Detarium senegalense (Detah) and Erythrophleum guineense (Tali). In the estuary, the Rhyzophora and Avicenniia mangroves take over an area of about 100,000 ha.

That of the middle Casamance is characterized by formations of the Sudano-Guinean type dominated by Daniellia oliveri, Pterocarpus erinaceus and Bombax costatum. By account in upper Casamance, the vegetation is marked by stands with Sudano-Guinean affinity which thin out as one progresses towards the East. Four species predominate in the tree stratum Bombax costatum, Pterocarpus erinaceus, Daniellia oliveri, Cordyla pinnata, with an undergrowth composed of combretaceae and Terminalia macroptera (Wolosa).

Sampling and data collection

Sampling was carried out in four farms (Diatock and Oussouye) located in the natural Casamance region and (Santhie and Khay) in the Thies region. In each farm, we chose lemon, orange, mandarin and grapefruit which are host plants for *R. invadens*. In each plant, ten (10) specimens were collected. This made it possible to have 80 specimens in Casamance and 80 other specimens in Thies. Specimens were coded, taking into account both the area and the type of plant from which they were collected. The data are grouped in Table 1 below. In general, genetic analysis of *R. invadens* larvae is destructive. Indeed, it requires the prior death of individuals and their fixation on alcohol (Osafune *et al.*, 2005).

Legend: CDC: Casamance-Diatock-Citron; CDP: Casamance-Diatock-Pamplemousse; CDM: Casamance-Diatock-Mandarine; CDO: Casamance-Diatock-Orange; COC: Casamance-Oussouye-Citron; COP: Casamance-Oussouye-Pamplemousse; COM: Casamance-Oussouye-Mandarine; COO: Casamance-Oussouye-Orange; TSC: Thiès-Santhie-Citron; TSP: Thiès-Santhie-Grapefruit; TSM: Thiès-Santhie-Mandarine; TSO: Thies-Santhie-Orange; TKC: Thiès-Khay-Citron; TSP: Thiès-Khay-Pamplemousse; TKM: Thies-Khay-Mandarin; TKO: Thies-Khay-Orange

Genetic study

Extraction of DNA from R. invadens

R. invadens DNA extractions were performed using the Qiagen DNeasy Tissue Kit method. The insect was dissected. Only the thorax and the legs were used for the extraction. They were placed in a 1.5 ml tube to which 180 µl of ATL buffer allowing tissue lysis and 20 µl of proteinase K are added to degrade all the proteins after incubation at 55° C. for 3 hours. In case the sample was not completely digested, the mixture was centrifuged and the liquid was transferred to a new tube to remove cellular debris. The next step was to add buffer AL allowing cell lysis to vortex immediately, then to incubate the samples at 70° for 10 minutes. After this incubation, 96-100% ethanol was added to the mixture and deposited in a column, then centrifuged at 13,000 rpm for one minute, thus allowing the DNA to be retained at the level of the membrane of the column. The DNA (negatively charged) was adsorbed on the membrane of the column (positively charged) by ionic interaction. Proteins, lipids and polysaccharides have not been retained by the membrane and will precipitate to the bottom of the collection tubes, which will be discarded. The DNA fixed on the column was washed by successively adding buffer AW1 and AW2 (500 µl each), which pass through the membrane by centrifugation at 13000 rpm for 1 minute and 3 minutes, respectively. The elution was carried out in two steps: each column was placed in a 1.5ml tube. Elution buffer AE (50 µl) and (30 µl) were deposited directly on the membrane. After incubation at room temperature for 1 minute, the DNA was detached from the membrane by centrifugation at 13000 rpm for 1 minute. Visualization of DNA fragments was performed by electrophoresis on 1.5% agarose gel in 0.5x TAE buffer, stained with ethidium bromide.

PCR (polymerase chain reaction) of cytochrome b

Choice of gene (cytochrome b)

Cytochrome B, involved in the cellular oxidation chains of all living beings, is a good marker for phylogenetic studies. It is considered one of the most used genes for phylogeny and is probably the best-known mitochondrial gene with respect to the structure and function of the protein produced. The vast majority of studies aimed at tracing the history of populations over the past two decades have been based on the analysis of variations in mitochondrial DNA. In addition, it is a highly polymorphic

maternally transmitted gene. It contains either slow and fast-changing codon positions, as well as several conservative and variable regions or general domains. Therefore, this gene has been used for a wide variety of systematic questions, from "deep" phylogeny to population and recent levels of divergence. In addition, mitochondrial DNA, whose transmission is uniparental (by the mother), has an important privilege for studying evolution because its mutation rate is high, it has no meiotic recombination and its variations are not, therefore, due only to cumulative mutations (no interbreeding). Its variation is, therefore, slow and also lends itself very well to the calculation of genetic distance over relatively short periods.

PCR

PCR was performed on fragments of the mitochondrial gene encoding cytochrome b. This gene was amplified using primers CB1 (5'-TAT GTA CTA CCA TGA GGA CAA ATA TC-3') and CB2 (5'-ATT ACA CCT CCT AAT TTA TTAGGA AT-3'). The gene has been shown to be polymorphic and discriminating in insects in previous studies (Sembene, 2000). Amplification was performed in a 25 µl reaction volume containing 18.3 µl water, 2.5 µl 10x buffer, 1 µl additional MgCl2, 0.5 µl dNTP, 0.25 µl each primer , 0.2 µl of Taq polymerase and 2 µl of DNA extract. It was made by the repetition of cycles which ensures a multiplication by 2 of the target DNA at each cycle. It was carried out in equipment called a thermocycler with the following amplification conditions: initial denaturation at 94°C for three minutes, followed by 35 cycles which are repeated of denaturation at 94°C for 1 minute, hybridization at 47° C for 1 minute and elongation of the complementary DNA strand at 72°C for 1 minute, a final elongation at 72°C for 10 minutes completes the PCR (Appendix II.1). Visualization of DNA fragments was done by electrophoresis on 1.5% agarose gel in 0.5x TAE buffer, stained with ethidium bromide. The size of the fragments was determined using a molecular weight (MW) marker composed of several DNA fragments of known size which were deposited in the gel and migrated together with the samples. For each individual, two 25 µl PCRs were carried out for sequencing purposes.

Sequencing

The sequencing was carried out by the company Macrogen in South Korea. We have sequenced a portion of the mitochondrial cytochrome b gene which is of great interest. This technique consists of determining the nucleotide sequence of a DNA fragment. It makes it possible, by comparing the sequences of the same gene in different individuals of the same species or of different species, to highlight point mutations. Sequencing was based on a specific PCR reaction using, in addition to the usual compounds (matrix DNA, polymerase, primers, dNTPs, Mg^{2+}), modified nucleotides: dideoxyribonucleotides (ddNTPs). These ddNTPs have the particularity of being coupled to fluorochromes: ddATP-green, ddTTP-red, ddCTP-blue and ddGTP-yellow (in black on the electropherogram) but also of not having an OH group at the 3 end ' deoxyribose. The incorporation of these ddNTPs by the polymerase therefore blocks the elongation of the complementary DNA molecule being copied; which generates fragments of different sizes. The fragments of different sizes thus synthesized were then separated by electrophoresis on acrylamide gel (from the smallest to the largest). The reading of the gel is carried out by the automatic scanning of a laser which makes it possible to detect the various fluorochromes coupled to the 4 ddNTPs.

Genetic analyzes

After sequencing the samples; the sequences obtained were first carefully corrected and aligned with BioEdit Version 7.2.0 software (Hall, 1999) which uses the Clustal-W algorithm (Thomson *et al.*, 1994). They have been realigned with the T-coffee database (Di Tommaso *et al.*, 2011) which uses the same algorithm to determine the homology between sites, define haplotypes and identify the different variants of the sequences obtained. They were then submitted to databases in which they were realigned and compared to a reference sequence. Once the alignment was done, individuals whose sequences did not show a

similarity greater than or equal to 95% with the reference sequence were simply excluded from the analyses.

Genetic diversity

The study of genetic variability has made it possible to highlight the genetic parameters which are the number of variable and invariable sites, the number of informative and non-informative sites, etc. These parameters are determined with the DnaSP software. Added to this are the haplotypic and nucleotide diversities (Nei, 1978) determined with the DnaSP Version 5.1.10 software (Rozas *et al*., 2010). A haplotype network was established by the Network software in order to distinguish individuals sharing the same nucleotide sequence and the existence of an association between haplotypes.

Differentiation and genetic structuring

The estimation of genetic differentiation requires, in what interests us in this study, two indices: the genetic distances of Nei intra/inter populations (Host plants) which account for the close relationships between individuals within a species and between species but also stability. They were determined using MEGA Version 6.05 software (Tamura *et al*., 2013). The values of Fst (genetic differentiation factor) by populations (localities) are estimated using Arlequin software version 3.5.1.3 (Excoffier *et al*., 2010). This index provides information on the differentiation and the effect of subdivision of populations. According to Wright (1978), the closer the Fst is to 1, the more the populations are genetically structured between them. On the other hand, the populations do not show genetic differences if the Fst is zero. A value of $P < 0.05$ was considered significant.

The molecular variance (AMOVA) was evaluated by ARLEQUIN V3.1 (Excoffier *et al*., 2010). It is based on a procedure that aims to maximize the total genetic variance associated with the difference between population groups according to locality.

Phylogenetic reconstruction

In order to determine the evolutionary relationships between the different populations of *Rastrococcus invadens* studied, a phylogenetic reconstruction based on molecular data was made. The demographic evolution parameters of the populations, such as the D of Tajima and the FS of Fu are determined by the harlequin software version 3.1. Tajima's D is a statistical test that aims to distinguish randomly evolving populations from those whose evolution takes into account other factors such as population migrations or natural selection. Fu's FS is based on the probability of having an expected number of haplotypes greater than or equal to the number observed in a sample drawn from a population of constant size. As for the elaboration of the mismatch distribution curves of the species, they were implemented by Dnasp.5.10.01.

They combine two indices that test the goodness of fit of the distribution; these are the SSD (sum of the squares of the deviations) and the Rag (irregularity index). The MEGA software version 5.05 of Tamura *et al*. (2013) made it possible to perform the phylogenetic reconstruction of the trees by the following three different methods:

Neighbor-Joining is based on the matrix of genetic distances of ecotypes (the Kimura 2-parameter distance) taken in pairs to model evolutionary processes;

Maximum Parsimony considers that a tree is optimal when its total length (number of steps necessary to explain the analyzed data set) is minimal. A consensus of all the selected trees is then achieved; the Maximum Likelihood makes it possible to test all the stories that could have generated the current dataset analyzed.

The tree using the Bayesian inference method was built using Mrbayes software version 3.2.6 x 64 (Huelsenbeck and Ronquist, 2001). It is a probabilistic method based on the calculation of posterior probabilities of phylogenetic trees by combining a prior probability with the likelihood function.

Results

After performing DNA extraction, PCR and sequencing, the sequences obtained were used for genetic analysis. Several parameters will be calculated, in particular, the parameters of genetic variability, genetic diversity, and genetic differentiation, as well as demographic evolution parameters, etc.

Table 1. Samples analyzed.

Codes	Plant species	Localities	Number of individuals	Region
CDC	Lemon	Diatock	10	Casamance
CDP	Grapefruit	Diatock	10	Casamance
CDM	Mandarin	Diatock	10	Casamance
CDO	Orange tree	Diatock	10	Casamance
COC	Lemon	Oussouye	10	Casamance
COP	Grapefruit	Oussouye	10	Casamance
COM	Mandarin	Oussouye	10	Casamance
COO	Orange tree	Oussouye	10	Casamance
TSC	Lemon	Santhie	10	Thies
TSP	Grapefruit	Santhie	10	Thies
TSM	Mandarin	Santhie	10	Thies
TSO	Orange tree	Santhie	10	Thies
TKC	Lemon	Khay	10	Thies
TKP	Grapefruit	Khay	10	Thies
TKM	Mandarin	Khay	10	Thies
TKO	Orange tree	Khay	10	Thies

Legend: CDC: Casamance-Diatock-Citron; CDP: Casamance-Diatock-Pamplemousse; CDM: Casamance-Diatock-Mandarine; CDO: Casamance-Diatock-Orange; COC: Casamance-Oussouye-Citron; COP: Casamance-Oussouye-Pamplemousse; COM: Casamance-Oussouye-Mandarine; COO: Casamance-Oussouye-Orange; TSC: Thiès-Santhie-Citron; TSP: Thiès-Santhie-Grapefruit; TSM: Thiès-Santhie-Mandarine; TSO: Thies-Santhie-Orange; TKC: Thiès-Khay-Citron; TSP: Thiès-Khay-Pamplemousse; TKM: Thies-Khay-Mandarin; TKO: Thies-Khay-Orange.

Genetic diversity

Basic parameter of genetic diversity

The sequences obtained after alignment show that there is neither deletion nor difference in length of the sequences. Forty sequences including 750 sites were noted in each locality and then aligned and cleaned. The number of invariable and variable sites varies according to the localities with a higher rate of polymorphism in individuals of *R. invadens* of the Lemon tree of Diatock, the Mandarin tree of Oussouye, the Orange tree and the Grapefruit tree of Santhie and at the level of the Grapefruit tree of Khay. The number of informative sites is also much greater there than in other localities. On the other hand, in the *R. invadens* individuals of the Mandarin tree and the Lemon tree of Khay, the Lemon tree of Santhie, the Lemon tree of Oussouye and the Mandarin tree of Diatock do not present any variant (Table 2.

Haplotypes

All the individuals sampled in the two agro-ecological zones are divided into 6 haplotypes (Table 3. Haplotype 5 is the majority with 30 individuals or 18.75% of the total population; it is also called the original haplotype, followed by haplotypes 1 and 6 with 29 individuals or 18.12%, haplotype 4 with 25 individuals, i.e., 15.62%, haplotype 3 with 24 individuals, i.e., 15% and finally haplotype 2 with 23 individuals, i.e., 14.37%.

The number of haplotypes taken separately shows that there are 6 and 5 haplotypes, respectively, in the regions of natural Casamance and Thies. In Casamance and Thies, the majority of haplotypes are 2 and 1, respectively (Fig. 1).

Haplotype network

Fig. 1 shows networks of haplotypes: ©d, © and d corresponding respectively to the global population, Casamance and Thies. Each ellipse represents a haplotype, the size of each of which is proportional to its frequency (Fig. 1).

Table 2. Genetic polymorphism of R. *invadens* populations.

Settings / Localities	Species plants	Number of Sequences	Number of Sites	Invariant sights	Variant sights	Non informative sites	informative Sites	Haplotypes
Diatock	Lemon	10	750	729	21	6	15	5
	Orange tree	10	750	741	9	3	6	3
	Grapefruit	10	750	740	10	0	10	2
	Mandarin	10	750	750	0	0	0	1
Oussouye	Lemon	10	750	750	0	0	0	1
	Orange tree	10	750	743	7	0	7	2
	Grapefruit	10	750	742	8	0	8	2
	Mandarin	10	750	735	15	1	14	3
Santhie	Lemon	10	750	750	0	0	0	1
	Orange tree	10	750	732	18	7	11	2
	Grapefruit	10	750	734	16	0	16	3
	Mandarin	10	750	740	10	0	10	2
Khay	Lemon	10	750	750	0	0	0	1
	Orange tree	10	750	740	10	0	10	3
	Grapefruit	10	750	732	18	7	11	3
	Mandarin	10	750	750	0	0	0	1

The lines existing between two circles represent a mutational step. The haplotype network is star-shaped in Casamance both in Thies and at ©d, revealing a rapidly expanding global population of *R. invadens* individuals.

Genetic diversity indices (Hd and Pi)

The determination of the genetic diversity indices of individuals of *Rastrococcus invadens* at the level of plant species and according to localities shows that everywhere there is high haplotypic diversity (Hd) and low nucleotide diversity (Pi) (Table 4.

This was also noted for the cytochrome b gene of the whole population (four localities) (Hd = 0.837 ± 0.004; Pi = 0.010 ± 0.0003).

Table 3. Genetic polymorphism of *R. invadens* populations.

Haplotypes	Host Plants
Hap_1	ROD RCD RCO RCO RCO RCO RCO RCO RCO RCO RCO RCO ROO ROO ROO RMO RCS RCS RCS RCS RCS RCS RCS RCS RCS RPS RPS RPS
Hap_2	ROD ROD ROD ROD ROD ROD ROD ROD ROO ROO ROO ROO ROO ROO ROO RPO RPO RPO RPS RPS RPS RPS RPS
Hap_3	ROD RMD RMD RMD RMD RMD RMD RMD RMD RMD RMD RCD RPO RPO RPO RPO RPO RPO RPO RMO RMO RMO RMO RMO
Hap_4	RCD RCD RCD RMO RMO RMO RMO RMK RMK RMK RMK RMK RMK RMK RMK RMK RMK RPK RPK RPK RPS RPS ROS ROS ROS
Hap_5	RCD RPD RPD RPD RPD RPD RPD RPD RPK ROK ROK ROK ROK ROK RCK RCK RCK RCK RCK RCK RCK RCK RCK ROS RMS RMS RMS RMS RMS
Hap_6	RCD RCD RCD RCD RPD RPD RPD RPK RPK RPK RPK RPK RPK ROK ROK ROK ROK ROK ROS ROS ROS ROS ROS ROS RMS RMS RMS RMS RMS

Legend: ROD: Rastrococcus from the Diatock Orange tree; RPD: Rastrococcus from the Diatock Grapefruit tree; RMD: Rastrococcus from the Diatock Mandarin tree; RCD: Rastrococcus from the Diatock lemon tree; ROO: Rastrococcus from the Oussouye orange tree; RPO: Rastrococcus from the Oussouye grapefruit tree; RMO: Rastrococcus from the Oussouye mandarin tree; RCO: Rastrococcus from the Oussouye lemon tree ;ROS: Rastrococcus from Santhie Orange;RPS:Rastrococcus from Santhie Grapefruit;RMS:Rastrococcus from Santhie Mandarin;RCS:Rastrococcus from Santhie Lemon;ROK:Rastrococcus from Khay Orange; RPK: *Rastrococcus* from Khay's Grapefruit; RMK: *Rastrococcus* from Khay's Mandarin; RCK: *Rastrococcus* from the Lemon tree of Khay.

Differentiation and Genetic Structuring

Differentiation

It requires two indices: the genetic distances of Nei within and between populations and the Fst. They were evaluated at the level of each locality.

Diatock

The analysis of intra-group genetic distances of *Rastrococcus invadens* individuals shows heterogeneity for ROD, RCD and RPD individuals (Table 5. On the other hand, only the intra-group genetic distance of RMD is homogeneous. Furthermore, the analysis of inter-group genetic distances reveals that there are differences between the groups studied. The values of the P. Value at the level of the inter-group analysis are all significant. The Fst value =0.532 shows that *Rastrococcus* individuals are well structured.

Table 4. Genetic diversity of *R. invadens* populations in the study areas and according to host plants.

Localities	Host Plants	Génétic diversity Indices		Within population	
		Hd	Pi	Hd	Pi
Diatock	Lemon	0.8±0.1	0.011±0.001	0.812±0.027	0.009±0.0007
	Orange tree	0.378±0.181	0.003±0.001		
	Grapefruit	0.467±0.132	0.006±0.001		
	Mandarin	0.000±0.000	0.000±0.000		
Oussouye	Lemon	0.000±0.000	0.000±0.000	0.733±0.028	0.007±0.0009
	Orange tree	0.467±0.132	0.004±0.001		
	Grapefruit	0.467±0.132	0.004±0.001		
	Mandarin	0.644±0.101	0.010±0.001		
Khay	Lemon	0.000±0.000	0.000±0.000	0.676±0.023	0.011±0.0004
	Orange tree	0.556±0.075	0.007±0.0009		
	Grapefruit	0.6±0.131	0.008±0.002		
	Mandarin	0.000±0.000	0.000±0.000		
Santhie	Lemon	0.000±0.000	0.000±0.000	0.785±0.03	0.009±0.0007
	Orange tree	0.6±0.131	0.008±0.002		
	Grapefruit	0.689±0.104	0.008±0.001		
	Mandarin	0.556±0.075	0.007±0.0009		

Oussouye

The analysis of intra-group genetic distances reveals heterogeneity for ROO, RPO and RMO individuals and homogeneity for RCO individuals. Inter-group genetic distances show that there are differences between the sequences of *Rastrococcus invadens* from Lemon, Orange, Mandarin and Grapefruit taken two by two (Table 6). The values of the P. Value and the Fst are all significant.

Khay

The determination of the intra-group distances of the different individuals of *Rastrococcus invadens* shows us that those of the Mandarin tree and the Lemon tree of Khay are homogeneous. On the other hand, those of Orange and Grapefruit are heterogeneous. The inter-group analysis shows that there are differences between the groups of *Rastrococcus invadens* studied.

Table 5. Genetic distance of Nei from *Rastrococcus inva*dens in the locality of Diatock.

Genetic Distance of Nei						Genetic differentiation
Intra-group		Inter-group				Fst
Host plants		ROD	RMD	RCD	RPD	
ROD	0.0037					0.532*
RMD	0.0000	0.0091+				
RCD	0.0117	0.0130+	0.0120+			
RPD	0.0064	0.0133+	0.0103+	0.0119+		

Caption: ROD: Rastrococcus from Diatock Orange tree; RMD: Rastrococcus from the Diatock Mandarin tree; RCD: Rastrococcus from the Lemon tree of Diatock; RPD: Rastrococcus from the Diatock grapefruit; +: P. Value significant; *: highly significant.

The values of the inter-group P. Value are significant except for the groups (RMK, RCK) and (RPK, ROK). The value of Fst=0.628 is highly significant for all individuals (Table 7.

Santhie

The determination of intra-group distances is heterogeneous for individuals of *Rastrococcus invadens* of Grapefruit, Orange and Mandarin and homogeneous for Lemon. The inter-group analysis shows differences between the groups studied. The P. Value is significant for all the groups taken two by two except for those of (ROS, RMS). The value of Fst= 0.417 is significant for all the sequences.

Global population

The analysis of intra-group and inter-group genetic distances shows heterogeneity for all groups of Rastrococcus invadens. The values of the P. Value are all significant except for the group (RMT, ROT).

The Fst=0.286 for all the groups studied shows a weak structuring of the individuals of Rastrococcus invadens (Table 9).

Table 6. Genetic distance of Nei from *Rastrococcus invadens* in the locality of Oussouye.

Genetic Distance of Nei						Genetic differentiation
Intra-group		Inter-group				Fst
Host plants		RCO	ROO	RPO	RMO	
RCO	0.0000					
ROO	0.0045	0.0067+				0.397*
RPO	0.0052	0.0057+	0.0071+			
RMO	0.0109	0.0093+	0.0120+	0.0097+		

Legend: RCO: Rastrococcus from the Lemon tree of Oussouye; ROO: Rastrococcus from Oussouye orange tree; RPO: Rastrococcus from the Oussouye grapefruit tree; RMO: Rastrococcus from the Mandarin of Oussouye; +: P. Value significant; *: highly significant.

Genetic structure

The analysis of variance (Table 10) reveals an insignificant and low percentage of variance between populations from different agro-ecological zones (28.64). On the other hand, it is significantly higher within the populations of the same agro-ecological zone (71.35).

Table 7. Genetic distance of Nei from *Rastrococcus invadens* in Khay locality.

Genetic Distance of Nei						Genetic differentiation
Intra-group		Inter-group				Fst
Host plants		RMK	RPK	ROK	RCK	
RMK	0.0000					
RPK	0.0094	0.0113+				0.628*
ROK	0.0077	0.0183+	0.0103-			
RCK	0.0000	0.0213-	0.0147+	0.0069+		

Legend: RMK: Rastrococcus from Mandarin of Khay; RPK: Rastrococcus from Khay's Grapefruit; ROK: Rastrococcus from Khay Orange tree; RCK: Rastrococcus from the Lemon tree of Khay; +: P. Value significant; -: P. Value not significant; *: highly significant.

Depending on the individuals of *Rastrococcus invadens* and the locality concerned, our results reveal positive values of D of Tajima and Fs of Fu. Fu's Fs and Tajima's D values are not significant with P. values well above 0.05. The sum of squared deviations (SSD) is significant for all individuals with p-values less than 0.05 except for the RMC with a P. Value = 0.06. The irregularity index is not significant for ROC and RMC individuals (Table 12).

Phylogenetic trees of individuals

All phylogenetic trees are rooted with B. dorsalis. Six groups are obtained at the level of each tree.

For the Neighbor Joining and Maximum Likelihood trees, we find the same individuals for each group with different bootstrap values. The Maximum Parsimony tree also forms 6 clusters supported by high bootstrap values.

Table 8. Genetic distance of Nei from *Rastrococcus invadens* in the locality of Santhie.

Genetic Distance of Nei						Genetic differentiation
Intra-group		Inter-group				Fst
Host plants		RCS	RPS	ROS	RMS	
RCS	0.0000					0.417*
RPS	0.0094	0.0084+				
ROS	0.0094	0.0129+	0.0131+			
RMS	0.0077	0.0096+	0.0135+	0.0103-		

Legend: RCS: Rastrococcus from the Lemon tree of Santhie; RPS: Rastrococcus from Santhie grapefruit; ROS: Rastrococcus from Orange Santhie; RMS: Rastrococcus from the Santhie Mandarin tree; +: P. Value significant; -: P. Value not significant; *: highly significant.

The Bayesian inference tree shows 6 clusters. Five of these six groups include individuals of *Rastrococcus invadens* from all the host plants studied and the other group includes only those from Grapefruit and Orange. The particularity of its trees is that they all have a group made up solely of individuals of *Rastrococcus invadens* from Casamance (Diatock and Oussouye).

Table 9. Nei genetic distance of *Rastrococcus invadens* from cytochrome b.

Genetic Distance of Nei									Genetic differentiation
Intragroup		Inter-group							
Host plants		ROC	RMC	RCC	RPC	RMT	RPT	ROT	RCT	Fst
ROC	0.004									
RMC	0.007	0.010+								
RCC	0.009	0.010+	0.009+							0.286
RPC	0.009	0.010+	0.009+	0.009+						
RMT	0.011	0.015+	0.014+	0.012+	0.014+					
RPT	0.011	0.010+	0.012+	0.010+	0.012+	0.012+				
ROT	0.009	0.013+	0.013+	0.010+	0.011+	0.011-	0.011+			
RCT	0.004	0.010+	0.009+	0.008+	0.007+	0.014+	0.012+	0.011+		

Legend: ROC: Rastrococcus from the Casamance orange tree; RMC: Rastrococcus from the Casamance Mandarin tree; RCC: Rastrococcus from the Casamance lemon tree; RPC: Rastrococcus from the Casamance grapefruit tree; RMT: Rastrococcus from the Mandarin tree of Thiès; RPT: Rastrococcus from the Grapefruit tree of Thiès; ROT: Rastrococcus from the Orange Tree of Thiès; RCT: Rastrococcus from the lemon tree of Thiès; +: P. Value significant; -: P. Value not significant; D. genetics: Genetic differentiation.

Discussion

The present study aims to contribute to the knowledge of the genetic structuring of populations of *Rastrococcus invadens* on citrus in Senegal. The results obtained with the mitochondrial cytochrome b gene show that the individuals of *R. invadens* encountered in Senegal contain different haplotypes. Indeed, the study of haplotypes shows us that there are at least 6 haplotypes of *R. invadens* in the different agro-ecological zones and at the level of Orange and Grapefruit. On the other hand, we have 5 haplotypes at the level of the Lemon tree and the Mandarin tree. Haplotype 5 is considered the majority and ancestral haplotype. In the region of natural Casamance, three groups of *R. invadens* are encountered at the level of the Orange tree: group 1, which is found only in Diatock, group 2 and 3, which are different but are found at times in Diatock and Oussouye. We also find three groups of *R. invadens* at Oranger de Thies. Group 1 was found only in Santhie, group 2 and 3 quite distinct and found in both Santhie and Khay. Of the 6 haplotypes of the global dataset, there are 6 groups of R. *invadens* at the level of the Orange tree. This means that the individuals of *R. invadens* from the orange tree in natural Casamance are different from those from Thies. This difference could be explained by the adaptability of individuals of *R. invadens* in Casamance because the insect was observed for the first time in Senegal in the Niayes area (Han *et al.*, 2007).

Table 10. Percentage of variances according to the source of variation in each locality.

Sources of Variation	Variance percentage
Between populations of different agro-ecological zones	28.64
Within populations of the same agro-ecological zone	71.35

Five groups of *R. invadens* are found at the Lemon de Casamance. Group 1 is present at the same time in Diatock and Oussouye. On the other hand, the four other very distinct are found only in Diatock. Moreover, only two groups are encountered in Thies. Group 1 is only in Santhie and group 2 is only in Khay. Among the 6 haplotypes, the individuals of *R. invadens* of the Lemon tree account for 5. This suggests that certain individuals of R. invadens of the Lemon tree of Thies can infest the Lemon tree of Casamance.

The genetic structuring of *R. invadens* for the case of the Mandarin tree reveals three groups, both in Casamance and in Thies. Group 1 is present in both Diatock and Oussouye. The very different groups 2 and 3 are encountered in Oussouye. Among the three groups of Thies: group 1 contains only individuals from Khay and groups 2 and 3 those from Santhie. Of the 6 haplotypes, the Mandarin presents 5. This means that some individuals of *R. invadens* from Thies are found in Casamance on the one hand, notably those from Oussouye and Santhie and, on the other hand, Diatock and Khay. According to Fall *et al.*, 2020 individuals belonging to a previously defined zone show more similarities with other individuals from neighboring agro-ecological zones.

Four groups of *R. invadens* are noted for the grapefruit of Casamance and Thies. Groups 1 and 2 are only encountered in Oussouye and groups 3 and 4 in Diatock. In Thies, groups 1 and 2 are only present in Santhie, group 3 contains individuals found both in Santhie and Khay and group 4 is represented only by those of Khay. The individuals of *R. invadens* which infest the Grapefruit tree are present at the level of the 6 haplotypes. However, the greatest diversity of individuals of *R. invadens* is noted in Grapefruit and Orange (6 haplotypes), unlike Lemon and Mandarin (5 haplotypes). This leads us to say that Grapefruit and Orange trees are the types of plants most affected by the threats of *R. invadens*. This also leads us to say further that they are the second refuge plants after the Mango tree. According to Fall *et al.* (2020), citrus fruits and other fruit-bearing plants are considered to be places of refuge for the insect when the mango tree completes its cycle.

Table 11. Demographic change indices.

D of Tajima	Fs of Fu
1.267	7.539
P. Value= 0.860	P. Value= 0.988

The particularity of the haplotype network is that each ellipse contains different host plants from different localities. In fact, in all the localities studied, citrus fruits have become cash crops. Commercialization of citrus fruits can lead to significant gene flow through the introduction of new individuals from border areas and rapid multiplication of the species through year-round food availability. Indeed, there is significant human movement in Senegal for the transport of plant material (fruits, vegetables from one area to another); which allows people to interact with each other. The populations of *R. invadens* present a high haplotypic diversity and a low nucleotide diversity as well at the level of the host plants, the localities and for the cyt-b.

According to Grant and Bowen (1998), this may correspond to rapid population growth and an accumulation of mutations from a population of low effective size. The results of the genetic differentiation show that the individuals of *R. invadens* are well structured in Diatock as well as in Oussouye.

Table 12. Demographic change indices for each group.

P.D / P.H	ROC	RMC	RCC	RPC	RMT	RPT	ROT	RCT
D of Tajima	0.499	0.614	0.194	1.280	2.287	1.215	1.284	2.763
P. Value	0.726	0.772	0.633	0.920	0.995	0.913	0.919	0.999
Fs of Fu	4.728	7.636	5.102	7.136	11.398	6.759	9.924	7.629
P. Value	0.974	0.993	0.971	0.992	0.999	0.986	0.998	0.993
SSD	0.167	0.164	0.135	0.093	0.203	0.057	0.231	0.352
P. Value	0.030	0.060	0.030	0.000	0.000	0.000	0.010	0.000
Rag	0.504	0.540	0.282	0.168	0.359	0.100	0.346	0.778
P. Value	0.450	0.410	0.000	0.000	0.000	0.000	0.000	0.040

Legend: ROC: *Rastrococcus* from the Casamance orange tree; RMC: *Rastrococcus* from the Casamance Mandarin tree; RCC: *Rastrococcus* from the Casamance lemon tree; RPC: *Rastrococcus* from the Casamance grapefruit tree; RMT: *Rastrococcus* from the Mandarin tree of Thies; RPT: *Rastrococcus* from the Grapefruit tree of Thies; ROT: *Rastrococcus* from the Orange Tree of Thies; RCT: *Rastrococcus* from the lemon tree of Thies; P.H: Host plants; P.D: demographic parameters.

This is explained by the high significance of the P. Value and by the high value of the Fst 0.532* and 0.379*, respectively. Indeed, according to Wright (1978), the closer the Fst is to 1, the more the populations are genetically structured between them. We note roughly the same context in Thies, particularly in Khay and Santhie, except that the individuals from Thies are less heterogeneous compared to those from Casamance. This further confirms that the

Thies region is the focus of citrus infestation by *R. invadens*. We can therefore say that there is a strong structuring of *R. invadens* individuals at locality level.

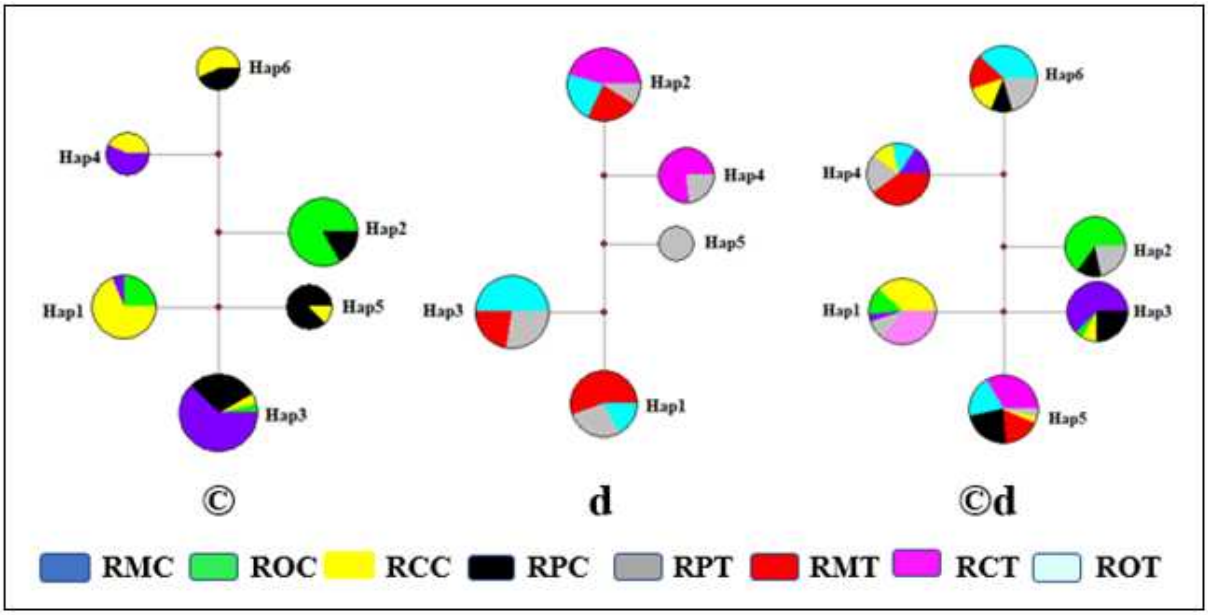

Fig. 1. Cytochrome b haplotype network of R. invadens populations encountered in the study areas (©d), © and d.

However, there is a weak genetic structuring of individuals of *R. invadens* as a whole despite the significance of the P. Value of the inter-group analyses. This low structuring is justified by the low value of Fst= 0.286. The absence of genetic differentiation in some individuals of *R. invadens* in Thies suggests from the first moments of the infestation, there was only one type of *R. invadens*. And it is according to the host plants and the agro-ecological zones that these differences between individuals of *R. invadens* appeared. The analysis of the molecular variable AMOVA made it possible to

see similarities between individuals of *R. invadens* in the different localities in terms of evolution. This is the case of Diatock and Khay where genetic differentiation takes place between individuals despite the fact that Khay is more homogeneous than Diatock. By counting those of Oussouye and Santhie, differentiation is observed within individuals of *R. invadens* as well as for populations of *R. invadens* as a whole. The non-significant values of Tajima's D and Fu's Fs and a multimodal curve of the distribution disparity testify to a stable overall population of *R. invadens*.

According to the sum of squared deviations (SSD) and the irregularity index (Rag), the populations of ROC, RPC, RCC, ROT, RCT, RPT and RMT have strongly significant P. Value values. This allows us to say that these populations are in demographic equilibrium or stable. Moreover, the population of RMC is in demographic expansion (P. Value not significant).

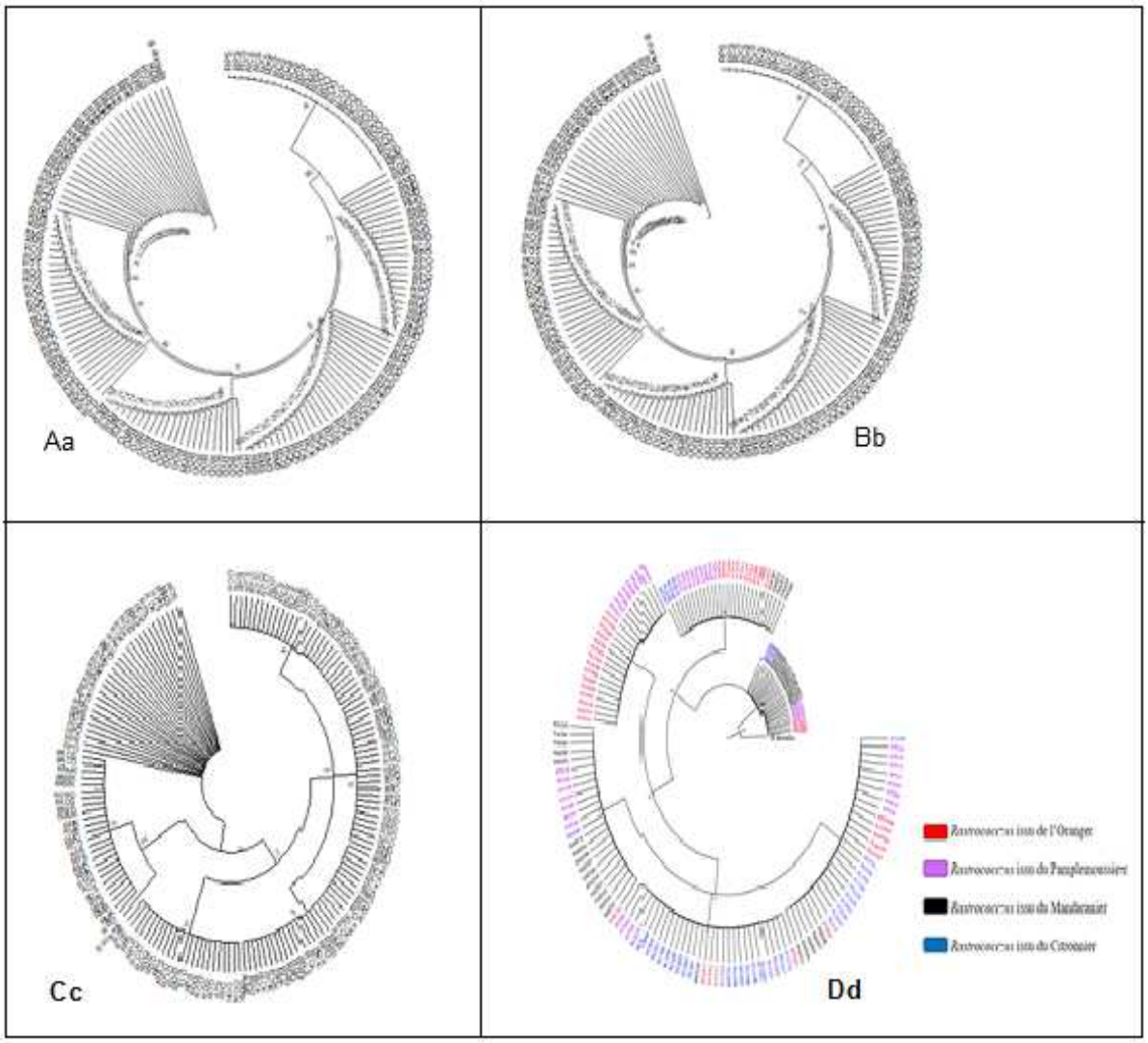

Fig. 2. Phylogenetic trees of individuals to highlight the structuring: Aa: Neighbor Joining, Bb: Maximum Likelihood, Cc: Maximum Parsimony and Dd: Bayesian tree.

Analysis of the Mismatch distribution curves of the difference numbers between haplotypes reveals a multimodal distribution, which indicates stable populations across the entire Cyt gene. B and in the different localities. This stability would be due to an adaptation of *R. invadens* to their environment by the presence perhaps of endocytobiotes. According to Hubert (1997), its endocytobiont beings play an important role in the accommodation of the host with respect to environmental variations.

The phylogenetic trees each form six groups and this confirms the results obtained previously. In each group we noticed the presence of individuals of *Rastrococcus invadens* from different host plants and different localities. This allows us to say that the structuring is done neither according to the host plants nor according to the localities.

Conclusion

The study of the genetic structure of populations of *R. invadens* has led to the conclusion that there is a single genus of *R. invadens*. Its evolution is that of a stable population. The Cyt gene. B has different tendencies within individuals of the same species. This is why we have listed different groups of *R. invadens*. However, the individuals of *R. invadens* are well structured with a total of 6 groups: one group which is common to the orange tree and the grapefruit tree and the five others are common to all the other host plants studied (the orange tree, the lemon tree, Mandarin and Grapefruit). Six groups of *R. invadens* on Orange and Grapefruit, five on Lemon and Mandarin. A strong structuring is also noted at the level of the localities. On the other hand, a weak structuring was noted in all the individuals of *R. invadens*. On the other hand, the development of the tree of haplotypes presents a grouping of haplotypes according to agro-ecological zones and host plants. The individuals of Diatock and Khay are differentiated in the same way on the one hand and, on the other hand, those of Oussouye and Santhie. Our results were obtained on fragments of the mitochondrial gene Cyt. B. However, we asked ourselves whether these results obtained are sufficient to confirm the existence of these six groups of *R. invadens* in citrus fruits. Beyond that, we thought of a much broader study:

First, extend the study with microsatellite markers to determine gene flow in agro-ecological zones and at the level of host plants.

Second, increase the size of the samples (agro-ecological zones, other types of citrus and localities) in order to have a better knowledge of the genetic structure of *R. invadens* populations.

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