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Identification and Genetic Characterisation of Populations of *Tuta absoluta* (Meyrick, 1917; Lepidoptera: Gelechidae) Destroyer of Tomato Farming in Senegal

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Abstract: In Senegal tomato jeopardiser *Tuta absoluta* (Meyrick 1917; Lepidoptera: Gelechiidae) causes serious losses in tomato production. This species can feed on all the green parts of the tomato and can cause up to 100% damage if not controlled. Currently, the most effective method of pest management is the use of chemicals. However, as it is generally noticed, excessive and unconscious use of chemicals leads to very harmful environmental pollution. The objective of this study is to identify the haplotypes of *Tuta absoluta* but also to see if agro-ecological zones and localities have contributed to genetically structure its populations. To achieve this goal, we adopted the PCR-Sequencing technique of the mitochondrial gene Cytochrome B of three populations of *T. absoluta* from three different localities in Senegal. The study has revealed the presence of 8 haplotypes and a population of *Tuta absoluta* in demographic balance. No significant genetic structuring has been noted between agro-ecological zones or localities. Moreover, phylogenetic reconstructions show no clustering either according to agro-ecological zones (Niayes and the Senegal River Valley), or according to localities. Nevertheless two clades were revealed by the latter, suggesting a multiple introduction of *T. absoluta* followed by an expansion to different geographical regions of the country by diffusion after its introduction.

Keywords: Agro Ecological Zones, Genetic Structuring, Localities, Haplotype, Jeopardiser

1. Introduction

Nowadays, agriculture is a leading sector in the world of economy. Horticulture (mainly fruits and vegetables) is one of the most dynamic area of the agricultural field in Senegal. Niayes represent a favourite zone for vegetable crops in Senegal because they meet ideal conditions. As previously reported by Naika *et al.* [24], tomatoes (*Solanum lycopersicum*) are among the most important vegetable crops in the world. In 2011, Chougar showed that tomato is a plant grown worldwide for its fruit. As mentioned in Trichpoulou & Lagio [35], it holds the second place behind potatoes in production and consumption. It is an annual plant originating from the Andes of South America and has since spread to Europe and other countries in Africa including Senegal [27]. It is a very important plant that provides a healthy and balanced diet and is very rich in vitamins and minerals; which means that it is consumed in different aspects: dried, ketchup, concentrate, jam juice… In addition, diseases caused by fungi, viruses, insects, nematodes are very often identified. As shown in Aroun *et al.* [2], tomato pests are very diverse; whiteflies, mites, mine flies are the main bio-aggressors of sheltered tomatoes. For our study, we will focus on the insect *Tuta absoluta*, pest of the tomato *Solanum lycopersicum*. This insect grows mainly on tomatoes but also on various other species of Solanaceae cultivated such as potato [26] and eggplant. It is a micro-lepidopteran causing significant

damage to tomato farming [7]. Therefore, it is currently the pest most feared by farmers both by the damage caused by caterpillars on leaves, stems and fruits and by the lack of effective means [2]. It is a lepidopteran that causes significant damage particularly in tomato crops, leaving mines on fruits, flowers, buds and stems [29]. This damage appear with whitish galleries dug by larvae on leaves. A larva can cause damage to several fruits of the same bouquet [28]. Indeed, *Tuta absoluta* has tomato (*Solanum lycopersicum*) as its preferred host, although it also grows on various Solanaceae species such as potato (*Solanum tuberosum*), aubergine (*Solanum melongena*), pear-melon or pépino (*Solanum muricatum*), peppers (*Capsicum sp*.), ferocious datura (*Datura ferox*) and glaucous or arborescent tobacco (*Nicotiana glauca*). *Tuta absoluta* was first observed in Europe in 2006 in Spain [1]. In 2008, it was reported in several countries around the Mediterranean and from 2009 in other European countries. It has been identified in Corsica and Provence and since then in the main tomato production regions. It is present in South America (Chile, Bolivia, Brazil, Colombia, Ecuador, Paraguay, Uruguay, Peru, Venezuela, and Argentina) in 2007, in several centres along the Mediterranean coast in the province of Valencia and on the island of Ibiza (Balearic Islands). In 2008, *T. absoluta* was reported in Morocco, Algeria and France (Corsica, Ajaccio region, Propriano, Bastia). It reached West Africa in 2012 through Senegal [27] and according to the DPV via plant material. It is known with certainty that trade and transfers of people between continents increase the possibilities of spreading the pest. Furthermore, it has been shown that *T. absoluta* can travel many kilometres by flying and being carried by the wind [22]. Despite its invasiveness and the extent of its polyphagia and damage, strong genetic homogeneity has been detected in its populations in the Mediterranean basin and South America, based on the analysis of mitochondrial and nuclear gene sequences COI and ITS [11]. The results of [15] showed high genetic homogeneity when analysing the genetic structure of four Argentine populations of *T. absoluta* compared to allozyme polymorphisms. It would then be interesting to characterize the Senegalese populations of *T. absoluta* in order to understand their degree of homogeneity or structuring but also factors controlling the distribution of the species. It is in this context that this study aims to know the genetic basis of the character or trait that explains its mode of invasion in Senegal.

2. Methodology

2.1. Biologic Material

Our study focuses on the larva of the lepidopteran pest *T. absoluta* and tomato seedlings.

2.2. Sampling

The sampling was carried out in two agro-ecological zones namely the Senegal River Valley and the Niayes area. In the Senegal River Valley, it took place on January 14th, 2019 in a village called Khatali located in the region of Saint-Louis. About 20 larvae of the insect in question were collected from a tomato field for pesticide use. In the Niayes area two localities were sampled: Gorom and Sacré-Coeur, including 10 larvae per locality. Each sample was coded using the first letter of the insect genus name and the first letter of the locality name. If the locality consists of two words, the first two letters of these two words are used in capital letters. Study populations were defined according to agro-ecological zones and localities.

Table 1. *Sample Synopsis.*

Localities	Agro-ecological Zones	Number of individuals	Sample code
Sacré-Cœur	Niayes	10	TSC
Gorom		10	TG
Saint-Louis	Senegal River Valley	20	TSL

TSL: *Tuta* Saint-Louis; TSC: *Tuta* Sacré-Cœur; TG; *Tuta* Gorom

2.3. Genetic Study

2.3.1. DNA Extraction

Extraction usually begins with the digestion of the *Tuta* larvae in order to individualize the cells using buffers and proteinase K. Thus, 180 µl of buffer ATL, 20 µl of proteinase K were poured into a tube and mixed with the dissected larva. The whole was vortexed for 15s and then incubated for 3h at temperature 55°C with interruptions every 30 min, to vortex. Then begins the cell lysis, which consists of perforating the nuclear and mitochondrial membrane so that the DNA molecule is released. Two hundred (200) µl of AL buffer was used then the samples were incubated at 70°C for 10 min. 96 ethanol was added and then centrifuged at 13000 turns for 1 min. Genomic DNA is absorbed by the silica membrane at the columns as the lysate passes through the membrane under the effect of centrifugal force. It follows the ionic purification. The AW1 buffer is used at 500 µl and then a centrifugation at 13000 rpm for 1 min. A centrifugation column is placed in a new tube by adding 500 µl of AW2 buffer followed by a centrifugation at 13000 rpm for 1 min. The last step is the elution, which involves placing a centrifugation column in an elution tube and then adding 50 µl of AE (elution solution) heated to 70°C before use. DNA was recovered by centrifugation.

2.3.2. PCR

The cytochrome b mitochondrial gene was targeted to perform polymorphism studies of insect DNA. This maternal-transmitted gene is of great interest because the vast majority of studies to trace the history and diversity of populations over the past two decades have been based on analysis of mitochondrial DNA variations [4]. The gene was found to be polymorphous and discriminant in insects in

previous studies [33]. A fragment of this mitochondrial genome was chosen as a marker to compare the genetic structure of *T. absoluta* populations. Several problems have been solved by analysing the genetic structure of the same portion of cytochrome b [6, 12-13, 21, 25].

After extraction, PCR was performed on the cytochrome b fragments. It consists of selective in vitro amplification of a particular sequence of matrix DNA by extension of two primers by a DNA polymerase, in the presence of deoxyribonucleotides (dNTP) and Mg2+ ions (magnesium). Amplification is performed by a repetition of cycles that ensures a doubling of the target DNA at each cycle. A master mix PCR is a ready-to-use solution containing Taq polymerase, dNTPs, MgCl2, and reaction buffer at optimal concentrations for PCR. The Master Mix contains all the components needed for the PCR, with the exception of the DNA sample to be amplified and the specific primers of the sequence to be amplified. The resulting mix consists of dNTP (0.5 µl), Tag polymerase (0.2 µl), water (17.8 µl), 10x buffer (2.5 µl), MgCl2 (1µl), forward primer (1µl) and reverse primer (1µl) and then concentrated DNA (1 µl) is added. It is performed in equipment called a thermal cycler under the following amplification conditions: initial denaturation for three minutes followed by 35 cycles (are repeated) denaturation at 94°C for 1 min, hybridization at 47°C for 1 min and a complementary DNA strand elongation at 72°C for 1 min. It was looped by a final elongation for 10 min.

2.3.3. Sequencing

Sequencing is based on the determination of the nucleotide sequence of a DNA fragment. It is a particular PCR reaction using, in addition to the usual compounds (matrix DNA, polymerase, primers, dNTP, Mg2+), modified nucleotides: didesoxyribonucleotides (ddNTP). Being in competition with dNTPs in the reaction medium, ddNTPs are randomly incorporated during the sequencing reaction, thus generating fragments of different sizes marked by different fluorescent colors: ddATP- green, ddTTP- red, ddCTP- blue and ddGTP-yellow.

2.4. Genetic Analysis

2.4.1. Sequence Analysis

Resulting sequences were manually aligned, verified and corrected using Bio Edit software version 7.2.5 [20] to determine the homology of the sites. These parameters were determined using software. Among them, the software DnaSP 5.10.01 [30] which made it possible to determine the number of sites (N), the sample size (n), variable sites such as singleton or non-informative sites and informative sites thriftily. The number of haplotypes (h) and other diversity parameters were estimated by the MEGA 7 software [34]. Thus, the rate of mutations (R), the types of mutations, that is to say the synonym mutations (Ks) and not synonyms (Kns), as well as the nature of the mutations were analysed by the software MEGA 7 version 7.0.14 [34]. The percentages of transitions and cross-cutting were calculated using the nucleotide substitution model. Synonymous and non-synonymous mutations are obtained using the Gojobori method. All these parameters were determined with the program DnaSP 5.10.01 [30]. However, nucleotide frequencies, amino acid frequencies and the nature of mutations were determined with MEGA 7 [34].

2.4.2. Basic Parameters of Genetic Diversity

Genetic Diversity Indices

We distinguish: haplotype diversity (Hd) and nucleotide diversity (Pi). Both were also determined with DnaSP software 5.10.01 [30]; based on the assumption that our population consists of three groups.

Genetic structuring

Structuring groups the parameters of genetic differentiation between populations which are two Fst (the degree of genetic differentiation) determined by DnaSP and D (the genetic distance) estimated by MEGA7 [34]. The values of Fst between populations can be evaluated with the Harlequin program version 3.5.1.3 [14]. The haplotype network is obtained using the Network 5.0.0.1 software [5].

AMOVA

AMOVA (Molecular Variance Analysis) analyses were performed using the Harlequin version 3.1 software [14] to determine the genetic structuring of populations according to a given factor.

2.5. Demographic Trends

The latter consists of genetic testing and analysis of Mismatch distribution.

Demogenetic tests are performed to distinguish sequences whose evolution follows an evolutionary model neutral to that evolving according to a non-random process. Tajima D parameters; D* and Fs of Fu [16] were estimated by DnaSP software version 5.10 [30]. Demo-genetic tests were performed to distinguish sequences (loci) whose evolution follows a neutral model from those evolving according to a non-random process. The aim is to compare the level of adjustment between the diversity observed at the loci and that expected under the hypothesis of a neutralist model (at the mutation-drift equilibrium).

2.6. Analysis of Mismatch Distribution

Mismatch distribution graphs are built using DnaSP Software version 5.10 [30]. The two indices which it associates in particular the SSD (Sum of Squares of Deviations) and the Rag (irregularity index) were estimated using the software Harlequin version 3.5.1.3 [14].

2.7. Phylogenetic Approach

The reconstruction of the phylogenetic trees with the two approaches (Neighbor-Joining, and Maximum Likelihood) were done using the MEGA 7 software [34]. The Neighbour-Joining method [31] is based on the matrix of the genetic distances of ecotypes (the distance of Kimura 2-parameter) taken two by two to model evolutionary processes. The Maximum Likelihood method (Felsenstein,

1981), using the HKY model and the Gamma law after a test with the MEGA 7 software [34], allowed to test all the stories that could have generated the current data set analysed. For these two possible approaches to tree reconstructions, the bootstrap test was used to testify to the robustness of the internal nodes that represent the ancestors. The degree of convergence of the chains can be verified by examining the evolution of the likelihood function during the course of the «cold» chain in order to determine the ignition period. Generations completed during this period are eliminated from subsequent analyses and estimates. Conservatively, the first 250,000 generations have been eliminated (25%) and inferences are then made over the next 750,000 generations. The reconstructions were rooted with a sequence of *Plutella xylostella*.

3. Results

3.1. Genetic Variability

After cleaning and alignment of sequences, no deletions or length differences were observed. A total of 25 DNA sequences each comprising 453 sites of which 431 sites are monomorphic and 22 of the polymorphic sites. Among these polymorphic sites, there are no single sites. All 22 polymorphic sites are informative thriftily. In these informative sites, the 21 have two variables and the rest consists of an informative site with three variables. The total number of mutations is 23 (Table 2). The presence of polymorphic sites implies the existence of mutations (transitions or cross-cutting) between the different sequences.

Table 2. *Parameters of genetic diversity.*

N	ETA	Size (Pb)	I	V	S	P	P2V	P3V
25	23	453	431	22	0	22	21	1

N: sample size; ETA: Total mutation number; I: invariable sites; V: variable sites; S: single sites; P: sites in thrift; P2V: thrift with two variables; P3V: thrift with three variables.

Table 3. *Percentage of transition and cross-cutting.*

	A	T/U	C	G
A	-	6.74	2.49	9.53
T/U	*8.92*	-	5.50	3.08
C	8.92	14.91	-	3.08
G	27.61	6.74	2.49	-

In Table 3 are recorded the frequencies of transitions (in bold) and cross-cutting (italics). Of all mutations, transitions are more numerous (57.55%) than cross-cutting (42.46%). In 14.91% of mutations cytosine replaces thymine. The latter replaces the first in 5.50% of substitutions. For transitions between puric bases, guanine replaces adenine in 27.61% and the last replaces the first in 9.53% of mutations. For those of transverse types guanine and adenine replace thymine in 6.74% and cytosine in 2.49%. Cytosine and thymine replace adenine in 8.92% and guanine in 3.08% of mutations.

There are eight haplotypes (h) (Table 4). In this table, the different haplotypes are distributed in descending order. The majority haplotype H6 consists of 9 individuals including 3 individuals of Saint-Louis, 4 individuals of Gorom and 2 individuals of Sacré-Coeur, followed by H3 (5 individuals) with individuals of Gorom and few individuals of Saint-Louis (1 individual) and Sacré-Cœur (1 individual). Haplotypes 1, 4, 5, 7 and 8 consist of two individuals each, unlike haplotype 2 which is unique.

Table 4. *Haplotype distribution.*

Haplotypes	Individuals number	Constituent individuals
H6	9	TSL6, TSL7, TSL9, TSC10, TG4, TG6, TG7, TG8, TSC5
H3	5	TSL3, TG1, TG2, TG3, TSC6
H1	2	TSL1, TSC2
H4	2	TSL4, TG10
H5	2	TSL5, TSC4
H7	2	TG5, TSC3
H8	2	TG9, TSC1
H2	1	TSL2

3.2. Genetic Diversity

Table 5. *Haplotype (Hd) and nucleotide (Pi) diversity in the total population.*

	Hd	Pi
Diversity indices	0.830	0.01082
Standard deviation	0.00299	0.0000150

Values of haplotype (Hd) and nucleotide (Pi) diversities in the total population are shown in Table 5. There are high haplotype diversity and low nucleotide diversity.

For all localities and agro-ecological zones, there is high haplotype diversity and low nucleotide diversity (see Tables 6 and 7).

Table 6. *Mean number of nucleotide differences (K), and nucleotide (Pi) and haplotype (Hd) diversities of localities.*

Localities	K	Pi	Hd
Saint-Louis	6.67	0.01472 P=0.84500	0.83333
Gorom	1.49	0.00329 P=0.83100	0.80000
Sacré-Cœur	8.47	0.01869 P=0.84100	1.00000

Table 7. *Mean number of nucleotide differences (K), and nucleotide (Pi) and haplotype (Hd) diversities of agro-ecological zones.*

Agro-ecologic zones	K	Pi	Hd
River Valley	6.66667	0.01472 P=0.84500	0.83333
Niayes	4.10000	0.00905 P=0.85000	0.85000

3.3. Genetic Structure

3.3.1. Genetic Distance

Genetic distance within populations is much greater in the Sacré-Coeur population of *T. absoluta* followed by Saint-Louis and finally Gorom 0.019, 0.015 and 0.003 respectively (Table 8).

Table 8. *Genetic distance within each locality.*

	Distance	Standard deviation
Saint-Louis	0.015	0.003
Gorom	0.003	0.002
Sacré-Cœur	0.019	0.004

The genetic distance between the localities is small: between Saint-Louis and Gorom (0.002), between Saint-Louis and Sacré-Coeur (0.003) and between Gorom and Sacré-Cœur (0.002). The same is true in agro-ecological zones (0.0022 0.0051 in the Senegal River Valley and 0.0013 0.0042 in Niayes) and between agro-ecological zones (0.0053).

There was non-significant genetic differentiation between localities (P>0.05) (Table 9).

Table 9. *Tsp between localities taken two by two.*

Fst P-values	Sacré-Cœur	Gorom
Gorom	0.04005 P = 0.25225	-
Saint-Louis	-0.14093 P = 0.90090	0.12767 P = 0.09009

The Fst is also insignificant between the two agro-ecological zones that is to say Niayes and the Senegal River Valley (-0.05212; P-value = 0.69369).

3.3.2. Estimation of Genetic Variability by AMOVA

Results show that 98.72% of the variability is due to the difference between individuals in the same population and 1.28% between populations (Table 10).

Table 10. *AMOVA test between different localities.*

source of variation	d. f.	Sum of squares	variance component	percentage of variance	P-value
Between populations	2	5.906	0.03519 Va	1.28	
Within populations	19	51.367	2.70351 Vb	98.72	
Total	21	57.273	2.73870	-	
fixation indexes	Fst (between localities)		0.01285		0.35386

The Tsp is not significant because the P-value is greater than 0.05

3.4. Demographic Trends

3.4.1. Demogenetic Test

The value of D of Tajima and Fs of Fu are negative and not significant within the three localities (Table 11). The R2 is positive and not significant so we retain the hypothesis 0 which is a stability.

Table 11. *Demogenetic indices for each locality.*

Localities	D of Tajima	P-Value	Fs of Fu	P-Value	R2	P-value
Sl	-0.00576	0.51800	-1.05269	0.16400	0.15902	0.000
G	-0.63193	0.29100	-1.28426	0.12300	0.15859	0.000
Sc	-0.76043	0.28700	-1.21912	0.12800	0.16121	0.000
Global	-0.36062	0.38300	-1.35916	0.77400	0,.16027	0.000

The same trends were observed between agro-ecological zones for the D of Tajima which is negative and not significant (Table 12).

Table 12. *Demogenetic indices for each agro-ecological zone.*

Agro-ecologic zones	D of Tajima	P-Value	Fs of Fu	P-Value	R2	P-value
Niayes	-0.09660	0.56000	0.40303	0.43600	0.16055	0.0000
Senegal River Valley	-0.00576	0.51800	0.32887	0.46200	0.15902	0.000
Global	-0.36062	0.38300	-1.35916	0.77400	0.1243	0.37700

3.4.2. Analysis of «Mismatch Distribution» or Pairwise Difference

The Mismatch Population Distribution of *Tuta absoluta* shows multimodal curves (Figure 1). The null hypothesis is used. We have demographic stability.

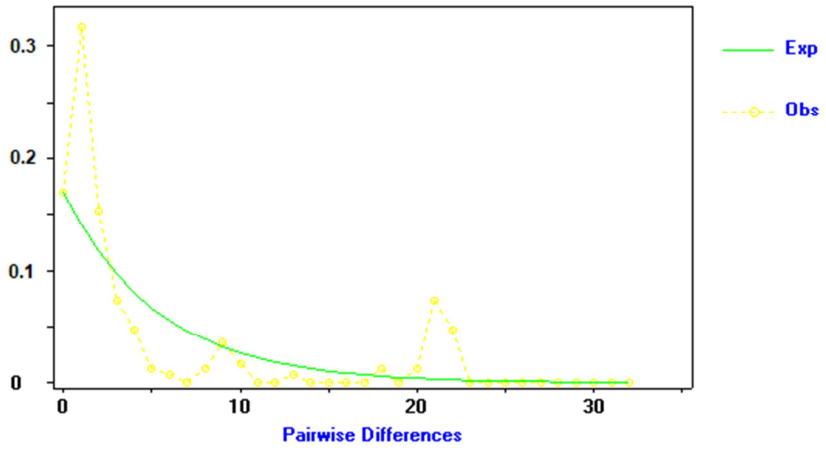

Figure 1. *Mismatch curve of overall population.*

3.5. Phylogenetic Approach

The phylogenetic trees reveal that there is no structuring either according to localities or according to agro-ecological zones. Nevertheless, they highlight two clades well supported by high bootstrap values for the Neighbour-Joining trees (Figure 2) and maximum likelihood and high posterior probability values for the Bayesian inference tree (Figure 3).

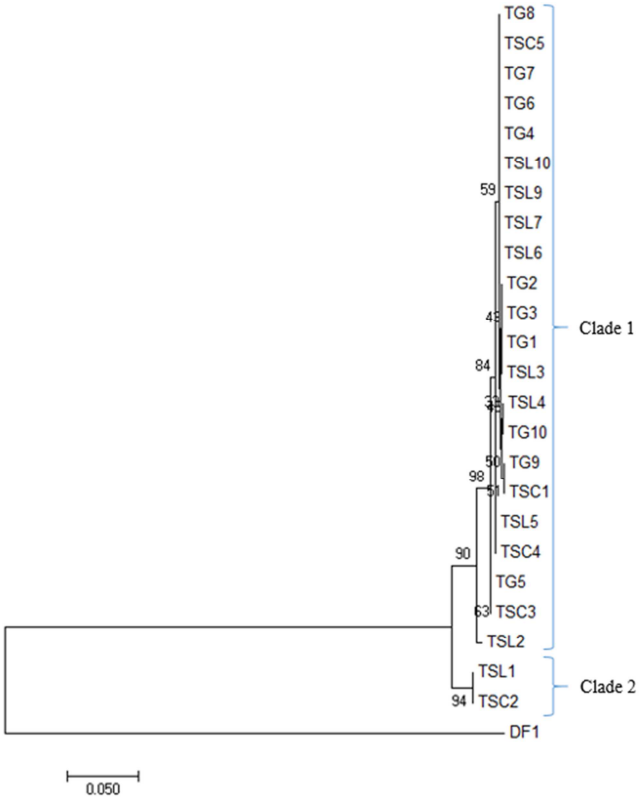

Figure 2. *Phylogram of T. absoluta individuals from Senegal using the Neighbour-Joining method (under the Kimura 2-parameter distance model).*

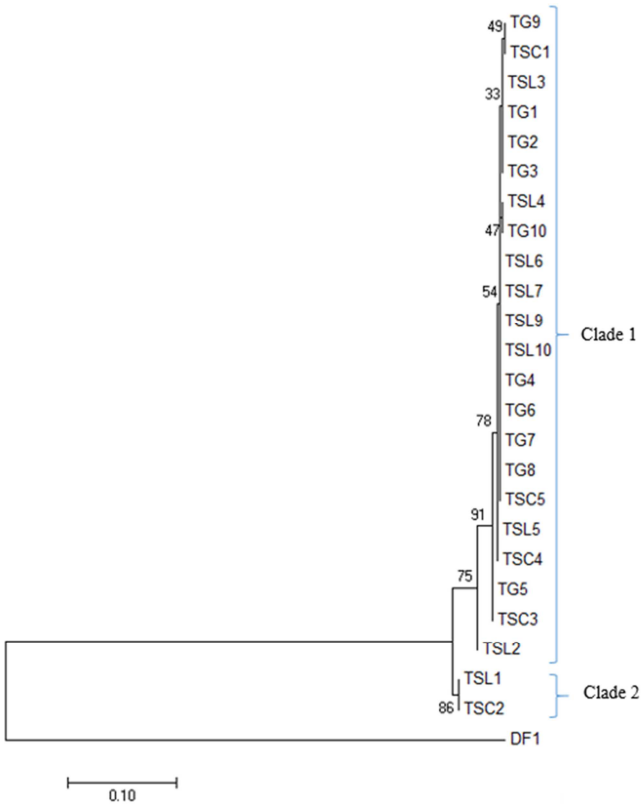

Figure 3. *Phylogramme of T. absoluta individuals from Senegal using the likelihood method obtained under the optimal HKY model and the Gamma law selected for this dataset.*

4. Discussion

The purpose of our study is to answer the scientific question whether the populations of *Tuta absoluta* differ according to localities (Sacré-Cœur, Gorom and Saint-Louis) and agro-ecological zones (Niayes and the Senegal River Valley) to know the genetic basis of the character or traits that explains its mode of invasion in Senegal. After sequencing a part of the

cytochrome b gene and careful alignment of the sequences obtained, we found a low degree of polymorphism (4.85% of variable sites on all sites of which 100% are informative in parsimony). This can be explained by the fact that individuals with the exception of two from Saint Louis (TSL1) and Sacré-Cœur (TSC2) are genetically close despite belonging to different localities and agro-ecological zones. This high genetic homogeneity was noted by Asma *et al.* [3] in populations of *T. absoluta* of Tunisian origin. It has also been detected in *T. absoluta* populations in India and Nepal [32]. Our results are also supported by those of Cifuentes *et al.* [11] which indicate a strong genetic homogeneity found in populations of *T. absoluta* from the Mediterranean basin and South America. Alternatively, selective scanning may be responsible for the absence of mitochondrial DNA variability. Eight (8) *T. absoluta* haplotypes were identified. This number is higher than that obtained by Asma *et al.* [3], a single haplotype on 21 specimens of Tunisian *T. absoluta* regardless of their host plants and localities. Haplotype diversity (Hd=0.830), nucleotide diversity (Pi=0.010) and the average proportion of nucleotide differences (4.90) in the total population are high relative to the diversity of COI (Hd) haplotypes of 0.015, nucleotides (Pi) 0.00008 and the mean proportion of nucleotide (K) differences of 0.045 found by Carvalho *et al.* [10]. Given that the introduction of the insect in Senegal is recent, we can still hypothesize that all these haplotypes would be introduced and that this invasion would be followed by a geographical expansion in the country. The genetic structure allows us to better explain the information cited above. The genetic distance of individuals within each locality, especially the locality of Gorom is not as great. The distance between localities is small (0.022) and that between the two areas, Niayes and the Senegal River Valley also (0.015). This may indicate that there is no structuring of populations according to localities and agro-ecological zones. The Fst between the different localities and agro-ecological zones is insignificant showing an absence of genetic differentiation. Our results are in agreement with those of [8] who noted a lack of genetic differentiation between Tunisian populations of *T. absoluta*. This could be explained by a large gene flow between populations. The analysis of molecular variances follows the same logic as that of the Fst. It reveals that most of the variability exists only between individuals (98.72%). This same trend was found by Bettaibi *et al.* [8] (71.85%). Phylogenetic trees reveal the clustering of the three populations in two clades, which shows a non-structuring of the populations of *T. absoluta* neither according to localities nor between agro-ecological zones. Despite the non-structuring of its populations according to agro-ecological zones, we note the presence of two clades each presenting the individuals of the two sampled zones. This result does not confirm that of Hadapad & Hire [19], which had a grouping of nine populations of *T. absoluta* under a single clade revealing no genetic variation within populations and showed great genetic homogeneity. Our results are in accordance with those of Guillemaud *et al.* [18] who used microsatellite markers and demonstrated that the indigenous population of *T. absoluta* in South America is far from genetically homogeneous. This would be explained by a multiple introduction of *T. absoluta* followed by an expansion to different geographical regions of the country by diffusion after its introduction.

5. Conclusion

This genetic study based on the sequencing of Cytochrome b of *Tuta absoluta* revealed the presence of at least eight (8) haplotypes in Senegal with the agro-ecological zones of Niayes and the Senegal River Valley. Low genetic diversity was noted in the study areas. It also reveals that the population of *T. absoluta* is in demographic balance. Small genetic distances between localities and agro-ecological zones were noted. Populations of *T. absoluta* show no tendency to be genetically structured either by locality or by agro-ecological zones. Despite the non-structuring of its populations, we note the presence of two clades, each presenting individuals of the two sampled areas. This would show the existence of multiple introductions of *T. absoluta* followed by expansion to different geographic regions by diffusion after its introduction.

More in-depth studies would provide a better understanding of relationships between populations, for example by:

(i) Diversifying sampling locations;

(ii) Taking into account the use of pesticides or not in the areas where the species is sampled.

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