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Impact of Rice Stem Borers and Identification of *Orseolia oryzivora* Harris & Gagné, 1982 (Diptera: Cecidomyiidae) Biotypes in the Southern Cameroon

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Abstract

The study compares the impact due to rice stem borers in two sites (Yaoundé and Ntui). It also shows the diversity of the African Rice Gall Midge (AfRGM) biotypes in southern Cameroon (Santchou, Ndop, Tonga, Ebolowa, Baïgom, Yaoundé and Ntui). The New Rice for Africa (NERICA) varieties 3, 8, 9 and 13 sown in Ntui were less attacked than those sown in Yaoundé. At both sites, damages ranged from 0.78% to 2.7%. In terms of diversity, the main stem-borer species were *O. oryzivora*, *Diopsis apicalis*, *D. longiconis* and *Chilo zacconius.* Molecular analyses of *Orseolia oryzivora* larvae collected in the localities of Santchou, Ndop, Tonga, Ebolowa, Baïgom and Yaoundé showed the existence of more than one *O. oryzivora* biotype in southern Cameroon's rice basins.

Keywords

Biotype, Larvae, NERICA, *Orseolia* sp, PCR

1. Introduction

Rice has become a strategic and priority product for food security in sub-Saharan

Africa [1]. Its consumption is increasing faster than the one of all other staples food due to high population growth, rapid urbanization and changes in dietary habits [2]. Rice is the first source of calories in West Africa and the third for the entire African continent [3]. In Cameroon, even though local production has increased rapidly after the 2007-2008 food crises, rice production remains below demand as in most African countries [1]. Demand for milled rice in Sub-Saharan Africa is expected to increase by 30 million tons by 2035, an increase of 130% [4]. NERICA has a great ecological plasticity [5] and increases the production in African countries in general and Cameroon in particular. However, rice cultivation is subject to various constraints, including diseases and insects pests [4]. Two main orders of stem borers, cause large yield losses to rice production in the intertropical regions of Africa. These are Lepidoptera: Noctuidae [6] [7]; Diptera: Diopsidae [7] [8] [9] and Cecidomyiidae [10] [11]. The system of insect pest was mainly based on their morphological and morphometric characters [12]. This technique has limits when it comes to distinguish cryptic, neighbouring or twin species [12]. The identification of differentiated populations in crop pests and their characterization using molecular markers is a necessary basis to define control strategies [13]. Some pest control programs have had very little success; this is partly due to a lack of knowledge about the genetic diversity of their populations and their history's evolution [14]. The AfRGM, *Orseolia oryzivora* is an indigenous pest species that causes serious damage to lowland rice crops in Burkina Faso, Nigeria, Mali, Sierra Leone, Cameroon and other sub-Saharan African countries [15] [16]. Host plant resistance is the single most effective means of controlling this insect pest. Screening for resistance over three years at the same location has provided evidence that there are large differences in resistance reactions to AfRGM between locations [17]. However, large differences suggest that there is more than one biotype present in West Africa and their distribution is unknown. In addition to the above, we need to know how virulent are these biotypes? Why and how resistant varieties withstand them? Work done by [14] [18] have revealed that the sequence characterized amplified region (SCAR) primers used in classifying the three *Orseolia* species (*O. oryzivora*, *O. bonzii* and *O. nwanzei*) in Nigeria could be useful for studies on biotypes and for field diagnostic purposes. The present work aims to find ways and means to improve pest management strategies in rice cultivation. We will evaluate the diversity and impact of stem borers and identify the biotypes of AfRGM using DNA fingerprinting techniques.

2. Materials and Methods

2.1. Presentation of the Different Sites

Data collection took place from March 2014 to August 2015 in eight sites in three agro-ecological zones of southern Cameroon: 1) Western Highlands, 2) South and transition zone between coastal plain and 3) highlands (**Figure 1**).

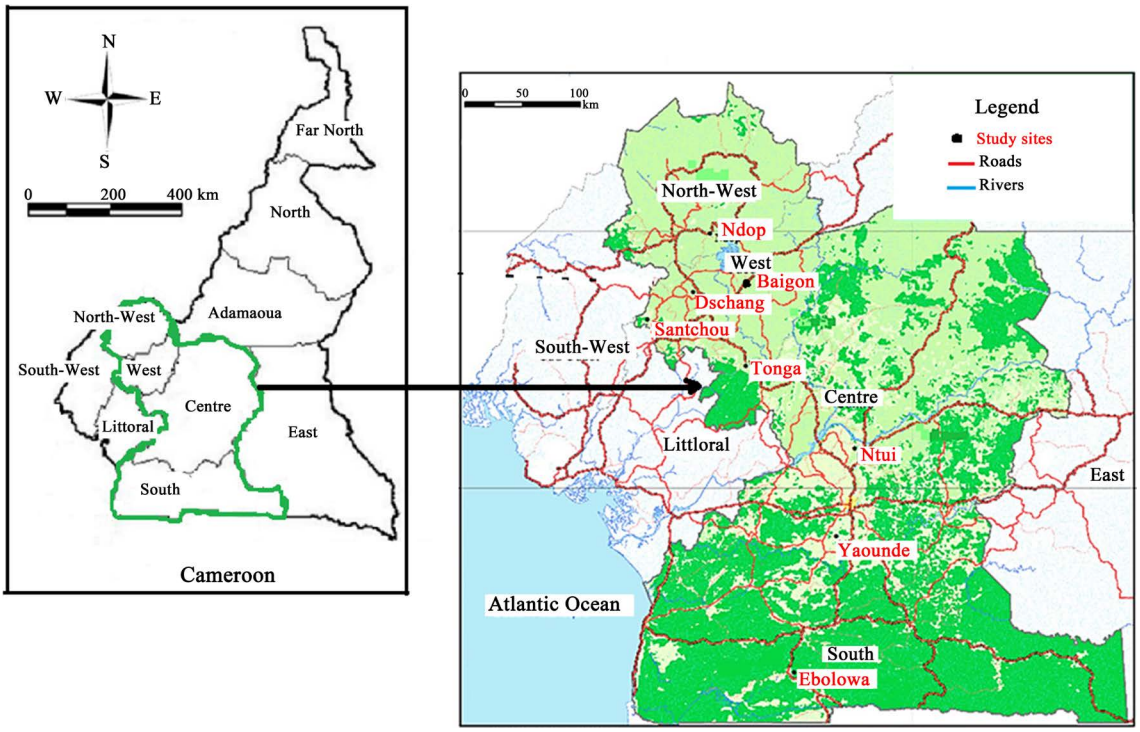


Figure 1. Sites localization.

2.1.1. Western Highlands

These sites are classified among the oldest rice-growing areas. Characterized by a humid climate with a unimodal rainfall, the agro-ecological zone of West Cameroon presents a very fertile agricultural land. Here, the data were collected in three localities: Dschang (05˚45'70.1"N - 10˚35'53"E, altitude: 1344 m) in the Menoua division, Ndop (06˚04'03.9"N - 010˚27'03.2"E, altitude: 1152 m) in the Ngo-Ketunjia division and Baïgom (04˚49'09.8"N - 011˚65'29.8"E, altitude: 1150 m) in the Noun division.

2.1.2. South Region

In this part of Cameroon, our research was carried out in three localities: Yaoundé-Nkolbisson (03˚51'57"N - 011˚27'11"E, altitude: 693 m) in the Mfoundi division, Ntui (04˚30'701"N - 011˚55'53"E., altitude: 509 m) in the Mbam-et-Kim division and Ebolowa (02˚56'52.1"N - 011˚07'9.7"E, altitude: 599 m) in the Mvilla division. In these localities, the climate is hot and humid, sub-tropical transition type, attenuated by altitude (Suchel, 1988). The annual temperature average is 23.5˚C contrasted between 16˚C and 31˚C depending on the season. Rainfall is 1650 mm per year [19]. The annual humidity average is 80% and varies during the day between 35% and 98%. Winds are wet and blow towards the southwest [19] [20].

2.1.3. Transition Zone between Coastal Plain and Western Highlands

In this region, we worked in two localities: Santchou (05˚15'47"N - 009˚58'10"E, altitude: 730 m) in the Menoua division and Tonga (04˚97'07"N - 010˚69'35"E,

altitude: 1344 m) in the Ndé division. The climate prevailing in Santchou and Tonga is an equatorial type, characterized by the alternation of dry seasons and rainy seasons. With an average temperature of 23.6˚C contrasted between 19 and 31˚C depending on the season and 2478 mm of rain per year.

2.2. Vegetal and Animal Material

The plant material consisted of six rice varieties: four NERICA varieties (3, 8, 9 and 13), Tonga and FKR 60 varieties. The animal material was *Orseolia* sp. insects. Their collection took place during the second growing season of 2014 (November-December) in the plantations of local producers on the sites of Santchou, Tonga, Ndop, Baïgom, Dschang, Ebolowa and on our experimental plots in Yaoundé and Ntui.

2.3. Diversity and Impact of Stem Borers

2.3.1. Planting, Fertilization, Maintenance and Sampling

Direct planting was carried out on manually ploughed soil and each variety has been planted on 10 m^2. The spacing was 20 cm between the columns and 20 cm on the line. The distance between two varieties was 50 cm. Two weeks after planting, 300 $kg{\cdot}ha^{-1}$ NPK (20-10-10) were applied; 65 $kg{\cdot}ha^{-1}$ of urea spread at 46% of panicle initiation, that is to say 60 - 70 days after sowing according to the life cycle of the variety and 35 $kg{\cdot}ha^{-1}$ of urea at the flowering stage. Three manual weeding was carried out, respectively 15, 30 and 60 days after planting. The sampling step was two weeks.

2.3.2. Evaluation Attacks

Stem borers attack rates were calculated by reporting the number of white heads observed for each variety over the number of total panicles for each variety.

$$\text{Attack rate} = \left(\text{Number of white heads}\right)/\left(\text{Number of panicles}\right)$$

2.4. Soil Physicochemical Parameters

Soil samples were collected using an auger at a depth of 0 - 20 cm in Ntui and Yaoundé. Parameters such as Humidity (%), Organic Material ($g{\cdot}kg^{-1}$), Organic Carbon ($g{\cdot}kg^{-1}$), Clay (%), Silt (%), Total Nitrogen ($g{\cdot}kg^{-1}$), pH, Sand (%) were recorded at the soil laboratory of the Agricultural Research Institute for Development (IRAD) at Yaoundé-Cameroon.

2.5. SCAR-PCR (Sequence Characterized Amplified Region-Polymerase Chain Reaction) Analysis

2.5.1. Identification and Conservation of Insects

The morphological identification, based on the dichotomous key [7] was first made. Forty seven insects, consisting of 46 larvae and 01 adult (obtained after raring) were collected from 04 localities in lowland and irrigated ecologies in Cameroon. These larvae and adult were preserved in absolute ethanol at −20˚C

inside 2 mL Eppendorf tubes before genomic DNA extraction [21]. The genomic DNA extraction and SCAR-PCR analysis were carried out at the laboratory.

2.5.2. Extraction of DNA with CTAB (Cetyl Trimethyl Ammonium Bromide)

The extraction of the DNA was carried out by adding 600 µL of CTAB buffer (CTAB 2%, 0.1 M TRIS/pH 8, 0.02 M EDTA/pH 8, 1.4 M NaCl) in each tube containing the biological material then, each sample was incubated in a water bath at 60˚C for 30 minutes. Then, 600 µL of a solution containing a mixture in respective proportions 24/1 of chloroform and isoamylic alcohol was added to each tube and the resulting mixture was homogenized for 10 minutes. Centrifugation at 8000 rpm for 15 minutes was carried out, three phases were then distinguished: the aqueous phase was removed and transferred to a new 1.5 mL Eppendorf tube labelled as the old one. A volume of 450 µL of isopropanol gal was added to the volume of the aqueous phase taken. The contents of the tube were homogenized once more (a few seconds this time) and incubated at −20˚C for at least 30 minutes, for the precipitation of the nucleic acids. Centrifugation at 13,000 rpm for 20 minutes and at 4˚C followed, allowing the DNA to settle into a pellet at the bottom of the tube. The supernatant liquid was removed and the pellet was washed with 1 mL of 70˚ ethanol and then centrifuged at 13,000 rpm for 15 minutes and at 4˚C. Once the alcohol evacuated, the DNA pellet was dried and then suspended in 20 µL of sterile water. The extracted DNA was stored at −20˚C until use.

2.5.3. Identification of Gall Midge Biotypes by SCAR-PCR

SCAR-PCR amplifies DNA sequences whose primers are designated from DNA sequenced fragments derived from RAPD (Random amplified polymorphic DNA) profiles.

Identification of the gall midge individuals was made by amplification of DNA sequences [14]. Each pair of primers used is specific to African Rice Gall Midge DNA (**Table 1**). The amplifications were carried out in a 25 µL reaction containing: the DNA extract, 2.5 µL, the 10 X TBE PCR buffer, 2.5 µL, 1 µL of the mixture of the deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), (10 mM), 1 µL of each primer of the SCAR primer pair considered concentrated at 10 Mm. 1 µL of $MgCl_2$ (25 mM), 0.1 µL of Taq polymerase (5 units/µL). The

Table 1. Sequences of primers used for identification of AfRGM species in Nigeria [14].

SCAR primer	Primer size	Sequence (5'-3')
OSSP-1	20	Reverse: GATTACGCCCAGGTCACTGT Forward: ATTACGCCCAGGTACCACAA
OSSP-5	20	Reverse: CGCCCAGGTACCATAACAAC Forward: AGTGATTACGCCCAGGTCAG
OSSP-6	20	Reverse: ACGCCCAGGTACCATAACAA Forward: AGTGATTACGCCCAGGTCAG

amplifications were done in a thermocycler and the amplification program included: an initial denaturation step of the DNA at 94˚C for 4 min, 35 cycles each containing a denaturation step of the DNA at 94˚C for 1 min, a primer hybridization step at 60˚C for 1 min and an elongation step at 72˚C for 2 min, a final extension step at 72˚C for 7 min.

After PCR, the amplification products were separated by electrophoresis on a 2% agarose gel. The migration was made under the effect of an electrophoresis generator, at 100 volts for 1 hour, and after this, the gel is stained in a 0.5 $mg{\cdot}mL^{-1}$ ethidium bromide solution. For the interpretation of the results, a molecular weight marker was added during the migration.

3. Results

3.1. Physico-Chemical Parameters of Soils at the Yaoundé and Ntui Sites

At these two sites, abiotic soil variables revealed similarities and differences for some parameters (Table 2). Thus, it appears that the percentage of clay in Yaoundé (57.91%) is almost double that of Ntui (29.23%). The same observation is made for the percentage of fine silt that is doubly lower in Ntui (7.95%) compared to Yaoundé (14.07%). The humidity of the Yaoundé (9.52%) was more than 03 times higher than the one of Ntui (2.98%). Parameters such as organic matter, organic carbon, total nitrogen, potassium and magnesium showed non-significant

Table 2. Physico-chemical parameters of the study sites.

Parameters	Ntui	Yaoundé
Humidity (%)	2.98	9.52
Organic material ($g{\cdot}kg^{-1}$)	20.80	19.33
Organic carbon (%) ($g{\cdot}kg^{-1}$)	12.06	11.21
Total nitrogen ($g{\cdot}kg^{-1}$)	1.22	1.90
pH (H_2O)	5.53	5.79
pH (KCl)	4.57	4.73
Clay (%)	29.32	57.91
Fine silt (%)	7.95	14.07
Coarse silt (%)	5.68	3.63
Fine sand (%)	27.08	14.74
Exchangeable bases (meq per 100 g soil)		
Na^+	0.22	0.18
K^+	0.31	0.40
Mg^{2+}	0.88	1.01
Ca^{2+}	2.98	3.66
CEC	7.90	11.92

fluctuations between the two sites. Overall, the soils at both sites were acidic.

3.2. Diversity and Impact of Stem Borers

3.2.1. Gall Midge

During this study, Gall Midges were observed in the plots at the second week after planting. The frequency of these symptoms has been relatively low. These symptoms were observed only at the Yaoundé site (Figure 2(a)).

3.2.2. White Heads

The first white heads were observed third week after planting on the Yaoundé site. Here, the most vulnerable varieties were: NERICA 3, NERICA 8, NERICA 9 and NERICA 13. The highest percentage of white heads (2.7%) was observed on the NERICA 3 variety, the lowest percentage (0.78%) was observed on the NERICA 9 variety (Figure 2(b)). Midge appeared at the vegetative stage of the host plant. At Ntui, the white heads did not appear until the ninth week after planting. FKR 60 had no white heads. In contrast, NERICA 3 had only one white head and NERICA 9 and 8 had the highest number of white heads (09 in total) (Figure 2(c)).

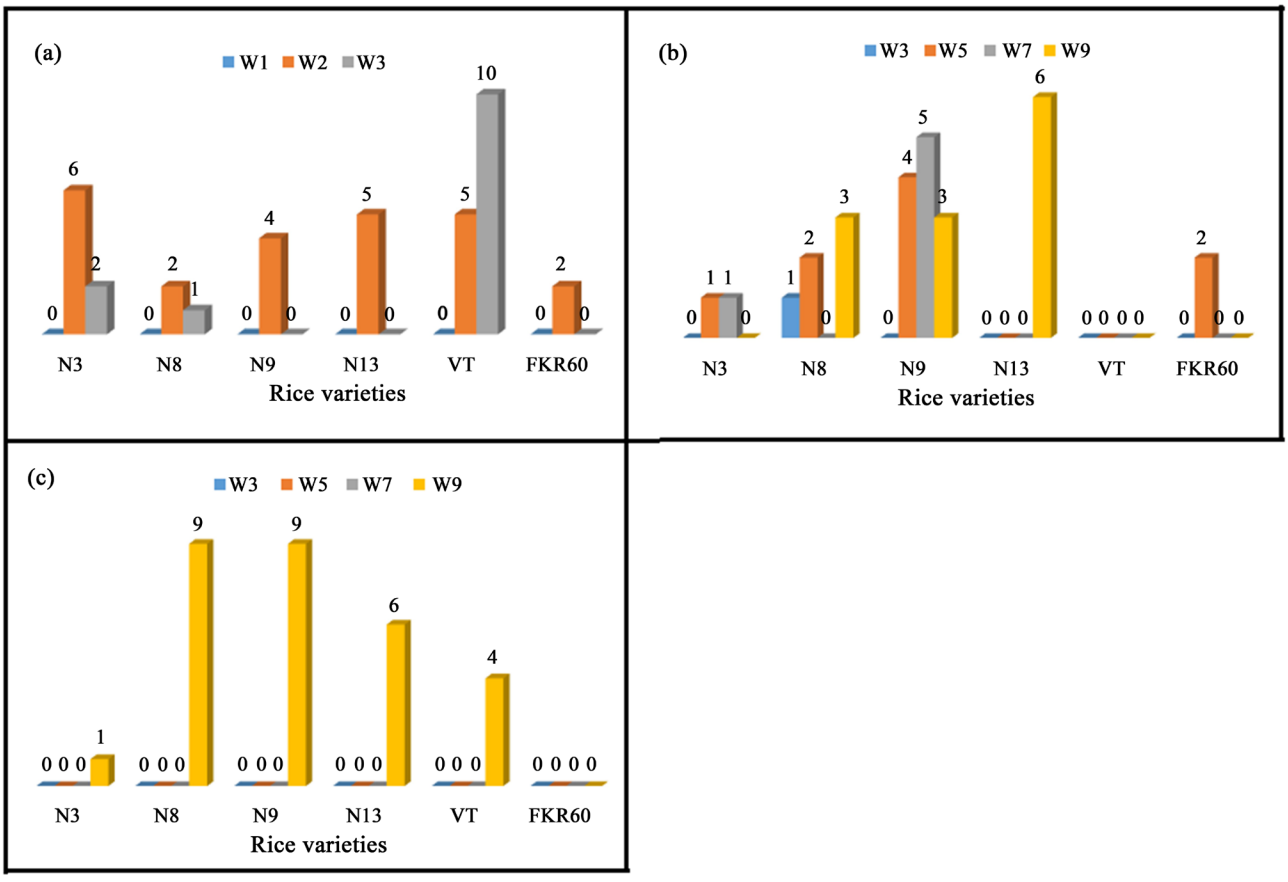


Figure 2. Weekly variations of White Head and Gall Midge abundances. (a) Gall Midge in Yaoundé; (b) White Head in Yaoundé; (c) White Heads in Ntui; FKR 60: FKR 60 variety; N (N3, N8, N9, and N13): NERICA; VT: Tonga variety; W (W1, W2, W3, W5, W7 and W9): Weeks.

In the Ntui site, only Lepidoptera larvae were present. We observed that many Lepidopteron larvae can coexist in the same stem, while Dipterans larvae such as *Diopsis* sp. and *Orseolia* sp. were usually solitary (only one larvae per rice stem).

In Ndop site, no white head was observed. Rice farmers told us not to know about the white head phenomenon. However, some gall midges were observed in the plots where the rice was still at the vegetative stage.

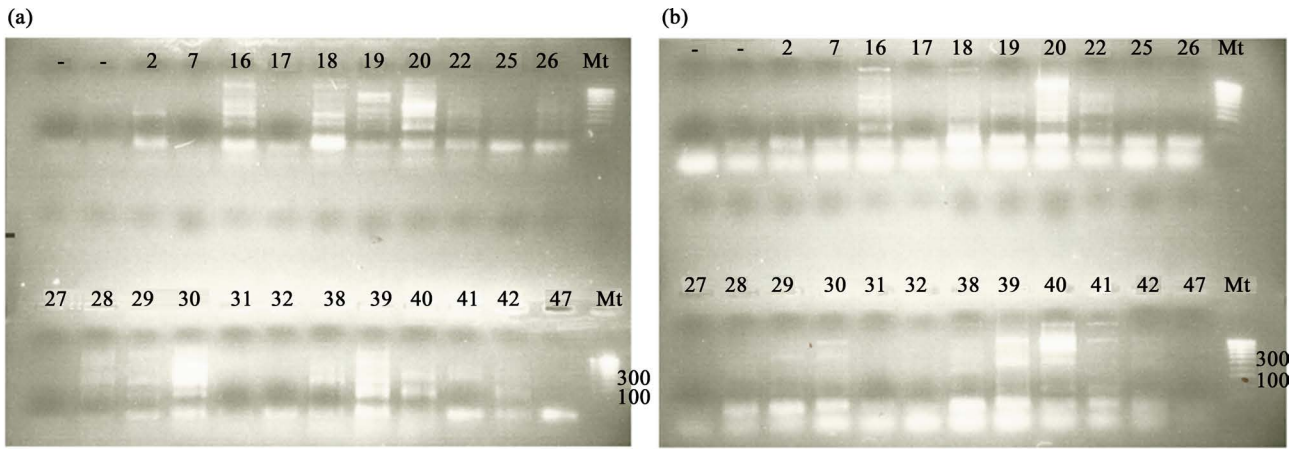
3.3. SCAR-PCR Analysis

From the six primer pairs tested, three successfully amplified our sample's DNA extracts. Successful amplification results in the appearance on the agarose gel of an observable ultraviolet band. We can determine the molecular weight of the protein (1000 bp) with the position of this band.

The DNA extracts corresponding to samples number 2, 16, 18, 19, 20, 22, 26, 28, 29, 30, 38, 39, 40, 41 and 42 were amplified by OSSP primer pair 5F/R (**Figure 3(a)**). The OSSP 1F/R primer pair amplified the DNA extracts corresponding to samples number 16, 18, 19, 20, 22, 25, 29, 30, 38, 39, 40, 41 and 42 (**Figure 3(b)**). The observation and interpretation of these bands obtained after amplification suggest that there is more than one biotype of the *Orseolia* genus in our samples.

From the larvae collected at Ebolowa, only the DNA extracts of samples no 22 and 47 were amplified (**Table 2**). Globally, only the DNA extracts from the larvae samples collected in the Ebolowa and Yaoundé sites were amplified (**Table 3**).

This result suggests that in Cameroon there are many biotypes of *O. orizyvora* among which the DNA extracts from Ebolowa and Yaoundé were amplified with three of the six primer pairs tested in Nigeria. At the Ebolowa site only DNA extracts from two individuals could be amplified. This suggests the potential existence of biotypes whose specific primer pairs are not among the six tested. This observation is also valid for individuals collected at the Yaoundé, Santchou and Ndop sites.



Mt: 1 kb molecular size marker; -: negative control.

Figure 3. DNA fingerprinting patterns of *Orseolia*. (a) By SCAR primer (OSSP 5F/R); (b) By SCAR primer (OSSP 1F/R).

Table 3. Larvae's DNA samples that amplified.

Sample's number	OSSP 1F/R	OSSP 6F/R	OSSP 5F/R	Sites
2		1	1	Yaoundé
7		1		Yaoundé
16	1	1	1	Yaoundé
17				Yaoundé
18	1		1	Yaoundé
19	1		1	Yaoundé
20	1		1	Yaoundé
22	1		1	Ebolowa
25	1			Yaoundé
26			1	Yaoundé
28		1	1	Yaoundé
29	1	1	1	Yaoundé
30	1	1	1	Yaoundé
31				Yaoundé
38	1		1	Yaoundé
39	1		1	Yaoundé
40	1		1	Yaoundé
41	1		1	Yaoundé
42	1		1	Yaoundé
47		1		Ebolowa

1 = amplified.

4. Discussion

4.1. Physico-Chemical Parameters

Biotic parameters measured at the Ntui and Yaoundé sites, showed differences for several variables, including nutrient ions and soil moisture content. This could be explained by the soil texture and climatic variables in these two localities.

Indeed, it has already been proven that enriching the soil with potassium improves rice yields [22] [23]. This enrichment could further reduce pesticide intake due to the negligible presence of pests, reduce yield losses and increase farmers' incomes for the sustainable development of rice. On the other hand, the incidence of midge increases with increasing levels of nitrogen fertilization [24]. The latter also showed that stem borers' attack was positively correlated with soil moisture and nitrogen content, hence the strong presence of white panicles and Yaoundé, compared to Ntui. In general, stem borers are still concentrated where food resources are most abundant [25]. The resistance of rice plants to stem borers in Ntui is thought to be related to a considerable range of phenotypic and/or genotypic characteristics [26]. However, environmental factors would be

responsible for the variation in the level of resistance from year to year or season to season [26] [27].

4.2. Impact of Stem Borers

Damage caused by stem borers in rice cultivation results in the appearance of dead hearts, midge in the vegetative stage of the plant and white heads in the flowering stage. Mechanically, the freshly hatched larvae bore into stem and feed internally causing death of central shoot dead hearts in vegetative stage and white head at flowering stage, respectively. This results in chaffy grains. The larvae feed on green tissue of leaf sheath [28].

The low rates of attacks in Ntui, observed at the end of flowering and the total absence of dead hearts during the vegetative stage could be related to climatic factors. It should be noted that, in Yaoundé, rice cultivation is still primitive and doesn't even exist in Ntui. These data confirm the hypothesis that *Orseolia* sp. expansion on the African continent is linked to the intensification of rice cultivation [29] [30]. These results would then confirm the performance of NERICA varieties against insects [5] [31] [32].

4.3. SCAR-PCR Analysis

After amplification and migration on agarose gel, observation of the bands revealed the presence of amplicons of different molecular weights, suggesting that there may be more than one biotype of *Orseolia* sp. in Cameroon; as already demonstrated in Nigeria [14]. DNA extracts from amplified samples appear to be genetically similar to those described in Nigeria [14] [18]. DNA extracts from several individuals, particularly those collected at Ebolowa, could not be amplified by the PCR technique. This could be due either to the low sensitivity of the primers, or the genetic material could be that of another species of the genus *Orseolia*, whose primers could not hybridize to DNA sequences.

Our results confirm the existence of differences between *Orseolia* biotypes at the molecular level. To date, three biotypes of *Orseolia* have been officially described in Africa [14] [18]. This study suggests the existence of various other biotypes to be characterized. The genus *Orseolia* is probably less species-rich in Africa than on the Asian continent, where 24 species have been described on the family Poaceae [33]. The existence of genetic variations between the three *Orseolia* biotypes, revealed by the RAPD and SCAR analysis, constitutes an effective discrimination tool that could be used to complement morphological traits to separate different biotypes present in Africa [14].

5. Conclusion

The objective of our study was to identify the *Orseolia oryzivora* biotypes and to study the impact of the main stem borer's species in the rice growing basins of southern Cameroon. Analysis of the physico-chemical parameters of soil samples taken in Yaoundé and Ntui showed the role that moisture, potassium and

nitrogen can play in the sensitivity of rice varieties to stem borers. Although NERICA varieties have been weakly attacked, no NERICA variety has complete resistance to stem borers. We observed larvae of *O. oryzivora* and *Chilo zacconius* in the attacked rice stalks. However, even if the variety NERICA 3 was attacked, only twenty samples out of forty-seven of the DNA extracts could be amplified. Our study shows that *Orseolia* sp. identified in Cameroon has several biotypes. For our future work, we intend to fully identify these biotypes using the high throughput sequencing technique.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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