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Identification and Differentiation of *Orseolia* Species in Nigeria as Revealed by SCAR-PCR Analysis

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Abstract: Identification and differentiation of *Orseolia* species in Nigeria was carried out using SCAR-PCR analysis. Twenty-three insects from 9 localities in Nigeria and three reference insects (*Orseolia bonzii*, *Orseolia nwanzei* and *Orseolia oryzivora*) were analyzed. Out of the 60 SCAR primers screened, only four produced clear amplified DNA fragments at annealing temperature of 55°C that differentiated all the 26 *Orseolia* species. Cluster analysis revealed two major insect genotypes (*OSG-1* and *OSG-2*). The *OSG-1* was further divided into two subgroups (*OSG-1a* and *OSG-1b*). Eleven insects were genotyped as *OSG-1a*, 14 as *OSG-1b* and one as *OSG-2*. Only NG1 and NG2 were identical among the insects of *OSG-1a* genotype. *OSG-1b* genotype produced two different groups of identical insects. While *O. bonzii* and *O. oryzivora* were genotyped as *OSG-1b* along with other twelve insects, only *O. nwanzei* was genotyped as *OSG-2*. *OSG-1b* genotype constitutes about 54% *O. bonzii* and *O. oryzivora* in Nigeria, *OSG-2* genotype represents 4% of *O. nwanzei* in Nigeria while *OSG-1a* genotype covers 42% of yet unknown *Orseolia* species in Nigeria. This information would strongly assist breeding programmes aiming at effective development of cultivars with durable resistance to African rice gall midge (AfRGM) in Nigeria.

Key words: Identification, differentiation, *Orseolia* species, *Orseolia bonzii*, *Orseolia nwanzei*, *Orseolia oryzivora*, SCAR-PCR based analysis, genotype, Nigeria

INTRODUCTION

The African Rice Gall Midge (AfRGM), *Orseolia oryzivora* Harris and Gagné (Diptera: Cecidomyiidae), is a serious insect pest of rainfed and irrigated lowland rice and is found

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only in Africa. It is an increasingly important insect pest of lowland rice in Nigeria (Ukwungwu *et al*., 1989). The pest was initially thought to be the same species as the Asian gall midge, *Orseolia oryzae* (Wood-Mason) but later separated from Asian rice gall midge based on the morphological characters of larvae, pupae and adults from the identical (Pathak and Dhaliwal, 1981; Waterhouse, 1993).

Orseolia oryzivora has been reported in thirteen West African countries including Burkina Faso, Mali, Nigeria, Sierra Leone and Cameroun (Ukwungwu *et al*., 1989; Ukwungwu and Joshi, 1992; Harris *et al*., 1999; Nwilene *et al*., 2002). The first major outbreak in Nigeria occurred in 1988 and affected about 50, 000 ha of rice. In the worst hit area in Abakaliki, Ebonyi State, about 45 to 80% tillers were infested on the worst affected fields. In the following year, similar damage was reported further South in the forest zone (Ukwungwu and Joshi, 1992). The insect has assumed a greater importance in recent years, especially after the introduction of high yielding varieties, changes in crop management practices and intensification of the production systems.

Field sampling has shown that AfRGM can increase very rapidly on many of the improved high-yielding rice varieties currently grown by farmers, thereby allowing for serious outbreaks to develop quickly. Thus, developing new rice varieties with higher levels of resistance to AfRGM is very important for improved management of the pest. However, this resistance can break down as a result of genetic change in the pest population (Katiyar *et al*., 2000; Lingaraj *et al*., 2008). Recent studies by the WARDA Integrated Pest Management Task Force have showed that the resistance of rice varieties to AfRGM differs markedly from one location to another. This is probably due to genetic differences between the AfRGM populations at different locations.

An understanding of this genetic variation amongst the population of the *O. oryzivora* is necessary for breeding programmes aimed at effective development of cultivars with durable resistance to AfRGM in Nigeria. Polymerase Chain Reaction (PCR) based genetic markers are widely used for molecular detection, genome mapping, map-based cloning and analysis of genetic variation in insects. These marker systems include random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990), Simple Sequence Repeats (SSR) (Tautz, 1989; Brown and Tanskley, 1996), Inter Simple Sequence Repeats (ISSR) polymerase chain reaction (Zeitkeinicz *et al*., 1994) and Amplified Fragment Length Polymorphisms (AFLP) (Vos *et al*., 1995). Sequence Characterized Amplified Regions (SCARs) are based on sequencing the polymorphic fragment derived from RAPD primers and designing longer primers that will specifically bind to this fragment (Nwilene *et al*., 2006). The present study aimed at using SCAR-PCR technique for the identification and differentiation of *Orseolia* species from Nigeria. The information from the study would strongly assist rice breeding programmes aiming at effective development of cultivars with durable resistance to African Rice Gall Midge (AfRGM) in Nigeria.

MATERIALS AND METHODS

Insect Collection and Storage

Twenty-three insects (Table 1), consisting of 2 larvae and 21 pupae were collected at random from 9 localities in lowland and irrigated ecologies in Nigeria. These larvae and pupae were preserved in absolute ethanol at -20°C inside 2 mL eppendorf tubes before genomic DNA extraction. The study was conducted between November 2008 and February 2009. The genomic DNA extraction and SCAR-PCR analysis were carried out at Central Biotechnology Laboratory, IITA Ibadan, Nigeria.

Table 1: Identity of *Orseolia* species used in the present study

Insect code	Life stage	Location	Site	Country	Host rice variety	Reference
NG1	Pupa	Ikwo Abakaliki	Agubia	Nigeria		This study
NG2	Pupa	Ikwo Abakaliki	Agubia	Nigeria		This study
NG3	Larva	Ikwo Abakaliki	Ogidiga	Nigeria	WITA 4	This study
NG4	Larva	Ikwo Abakaliki	Ogidiga	Nigeria	ITA 306	This study
NG5	Pupa	Badeggi	Chanchaga	Nigeria	NERICA 1	This study
NG6	Pupa	Badeggi	Badeggi	Nigeria	FARO 44	This study
NG7	Pupa	Badeggi	Emigara	Nigeria	FARO 44	This study
NG8	Pupa	Doko	Jima	Nigeria	FARO 52	This study
NG9	Pupa	Doko	Doko	Nigeria	Ebagichi	This study
NG10	Pupa	Edozhigi	Edozhigi	Nigeria	FARO 52	This study
NG11	Pupa	Edozhigi	Pichi	Nigeria	ROK 5	This study
NG12	Pupa	Edozhigi	Ejikokpata	Nigeria	FARO 44	This study
NG13	Pupa	Lemu	Anfani	Nigeria	FARO 52	This study
NG14	Pupa	Lemu	Ndakama	Nigeria	FARO 44	This study
NG15	Pupa	Lemu	Kuyizhi	Nigeria	Ebagichi	This study
NG16	Pupa	Gonagi	Gata	Nigeria	FARO 52	This study
NG17	Pupa	Gonagi	Kagba	Nigeria	Ebagichi	This study
NG18	Pupa	Guzan	Guzan	Nigeria	Ebagichi	This study
NG19	Pupa	Guzan	Kpabo	Nigeria	FARO 52	This study
NG20	Pupa	Kacha	Tswachi	Nigeria	FARO 44	This study
NG21	Pupa	Kacha	Loguma	Nigeria	FARO 52	This study
NG22	Pupa	Shendam	Shendam	Nigeria	Mahga	This study
NG23	Pupa	Shendam	Pandam	Nigeria	FARO 44	This study
Orseolia bonzii	Adult			Nigeria		Nwilene *et al.* (2006)
Orseolia nwanzei	Adult			Nigeria		Nwilene *et al.* (2006)
Orseolia oryzivora	Adult			Nigeria		Nwilene *et al.* (2006)

Genomic DNA Extraction

The 23 insects were then processed for DNA analyses according to Nicol *et al.* (1997) and Thottappilly *et al.* (1999), with some modification. Each was ground in liquid nitrogen, suspended in 200 µL of CTAB buffer (50 mM Tris (pH 8.0), 0.7 mM NaCl, 10 mM EDTA, 2% hexadecyltrimethylammonium bromide and 0.1% 2-mercaptoethanol), followed by the addition of 50 mL of 20% sodium dodecyl sulphate and incubated at 65°C for 30 min. The DNA was purified by two extractions with phenol: chloroform:isoamyl alcohol (24:25:1) and precipitated with -20°C absolute ethanol. After washing with 70% ethanol, the DNA was dried and re-suspended in 150 µL of sterile distilled water. The DNA concentration was measured using a DU-65 UV spectrophotometer (Beckman Instruments, Inc., Fullerto, California, USA) at 260 nm. DNA degradation was checked by electrophoresis on a 1% agarose gel in TAE (45 mM Tris-acetate, 1 mM EDTA (pH 8.0).

SCAR-PCR Analysis

The PCR analysis of genomic DNA from the 23 insects was carried out using SCAR primers developed by Nwilene *et al.* (2006). Genomic DNA from *O. bonzii*, *O. nwanzei* and *O. oryzivora* was included in the PCR analysis as control. Each of the designed SCAR primer pairs (one forward and one reverse SCAR primer) was tested on the insects genomic DNA. A total of sixty SCAR primer pairs were screened for their ability to amplify the insect genomic DNA. Four of these primers (Table 2) were found useful, since they gave polymorphism and were used in amplifying genomic DNA of all the 26 insects. Amplifications were performed in 25 mL reaction mixture consisting of genomic DNA, reaction buffer (Promega), 100 mM each of dATP, dCTP, dGTP and dTTP, 0.2 mM for each forward and reverse SCAR primer, 2.5 mM $MgCl_2$ and 1U of Taq polymerase (Boehringer, Germany). Two different annealing temperatures (60 and 55°C) were screened to determine the optimal annealing temperature. Amplification of genomic DNA with SCAR primers was done using

Table 2: Identity of SCAR primers that gave polymorphism after screening and used in the present study

SCAR primer	Sequence 5'-3'
OSSP5	Forward: ATTACGCCCAGGTACCACAA
OSSP7	Reverse: CGCCCAGGTACCATAACAAC
OSSP11	Forward: AGTGATTACGCCCAGGTCAG
OSSP6	Reverse: ACCGCACCGAATGATACCTA
OSSP14	Forward: CACTAGTGATTACGCCCAGGT
OSSP7	Reverse: CGCCCAGGTACCATAACAAC
OSSP16	Forward: TGATTACGCCCAGGTCGAT
OSSP1	Reverse: GATTACGCCCAGGTCACTGT

1 cycle of 94°C for 4 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min and 1 cycle of 72°C for 7 min. The amplification products were resolved by electrophoresis in a 1.4% agarose and stained in 0.5 mg mL^{-1} ethidium bromide solution. The presence and the absence of the SCAR band were visually scored and compared for each of the three *Orseolia* species. The banding patterns were photographed over UV light using a red filter.

Cluster Analysis

Positions of unequivocally scorable SCAR bands were transformed into a binary character matrix (1 for the presence and 0 for the absence of a band at a particular position). Pairwise distance matrices were compiled by the NTSYS-pc 2.0 software (Rohlf, 2000) using the Jaccard co-efficient of similarity (Ivchenko and Honov, 1998). Dendrograms were created by the unweighted pairgroup method arithmetic (UPGMA) average cluster analysis (Sneath and Sokal, 1973; Jakó *et al*., 2009).

RESULTS AND DISCUSSION

Identification and differentiation of *Orseolia* species in Nigeria was carried out using SCAR-PCR analysis. Of 60 SCAR primers screened, only four SCAR primers produced clear amplified DNA fragments at annealing temperature of 55°C. All the 26 insects were differentiated by SCAR-PCR analysis using the four SCAR primer pairs (Fig. 1-4). Cluster analysis revealed two major insect genotypes (*OSG-1* and *OSG-2*) (Fig. 5). The *OSG-1* genotype was further divided into two subgroups (*OSG-1a* and *OSG-1b*). Eleven insects were genotyped as *OSG-1a*, fourteen others were grouped as *OSG-1b* and one insect was genotyped as *OSG-2* (Fig. 1). Among the insects with the *OSG-1a* genotype, only NG1 and NG2 were identical. Two different groups of identical insects were identified among *OSG-1b* genotype. All the three reference insects (*O. bonzii*, *O. nwanzei* and *O. oryzivora*) were genetically distinct. While *O. bonzii* and *O. oryzivora* were genotyped as *OSG-1b* along with other twelve insects, only *O. nwanzei* was genotyped as *OSG-2*. *OSG-1b* genotype constitutes about 54% *O. bonzii* and *O. oryzivora* in Nigeria, *OSG-2* genotype represents 4% of *O. nwanzei* in Nigeria while *OSG-1a* genotype covers 42% of yet unknown *Orseolia* species in Nigeria (Table 3).

The existence of genetic variation among *Orseolia* species, as revealed by SCAR-PCR analyses, demonstrates its fingerprinting and diagnostic potential that could be used to identify and differentiate these insect species (Maruthi *et al*., 2007; Lu and Adang, 1996; Kakouli-Duarte *et al*., 2001; Armstrong *et al*., 1997; Behura *et al*., 1999). The use of genetic markers to efficiently and effectively identify genetic variations in the species is important in the management of the insects (Maruthi *et al*., 2007; Lu and Adang, 1996; Kakouli-Duarte *et al*., 2001; Armstrong *et al*., 1997; Behura *et al*., 1999). Insect classification and genetic relationships are important issues for entomologists working on host plant

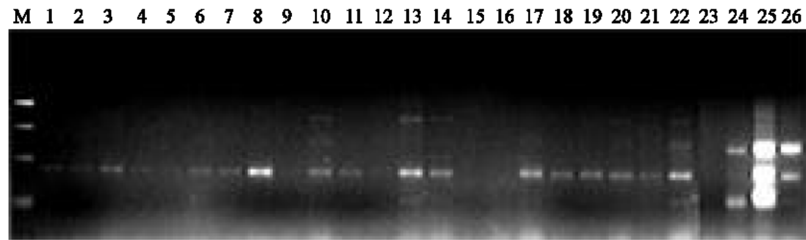

Fig. 1: The DNA fingerprinting patterns of *Orseolia* species using SCAR primer (OSSP5 forward: 5'ATTACGCCCAGGTACCACAA3'; OSSP7 reverse: 5'CGCCCAGGTAC CATAACAAC3'), M: 1 kb molecular size marker

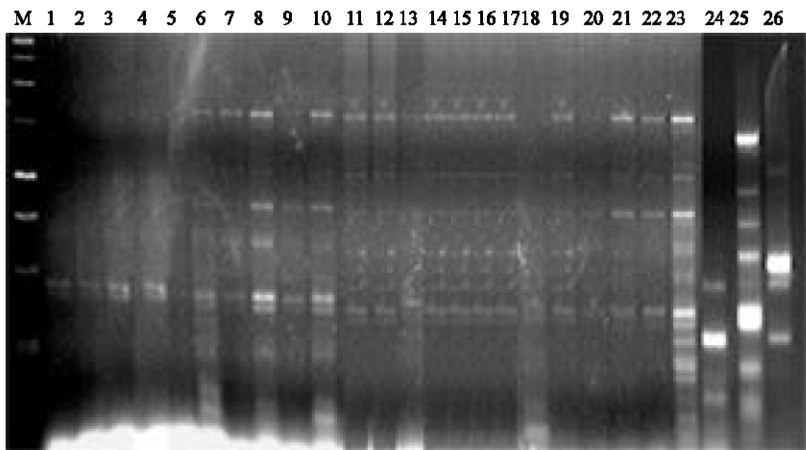

Fig. 2: The DNA fingerprinting patterns of *Orseolia* species using SCAR primer (OSSP11 forward: 5'AGTGATTACGCCCAGGTCAG3'; OSSP6 reverse: 5'ACCGCACCGAAT GATACCTA3'), M: 1 kb molecular size marker

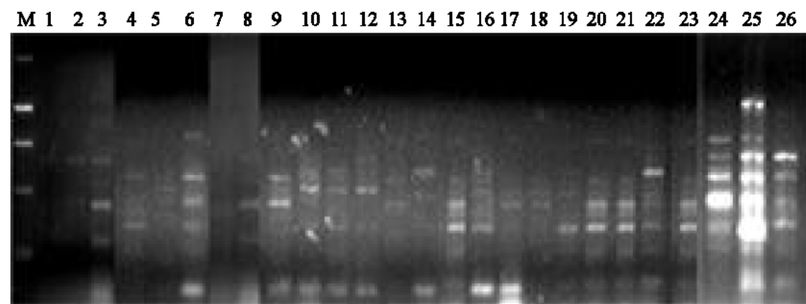

Fig. 3: The DNA fingerprinting patterns of *Orseolia* species using SCAR primer (OSSP14 forward: 5'CACTAGTGATTACGCCCAGGT3';OSSP7 reverse: 5'CGCCCAGGTACC ATAACAAC3'), M: 1 kb molecular size marker

resistance and biological control (Maruthi *et al*., 2007; Lu and Adang, 1996; Kakouli-Duarte *et al*., 2001; Armstrong *et al*., 1997; Behura *et al*., 1999). The application of SCARs as demonstrated in this study seems very useful in this regard.

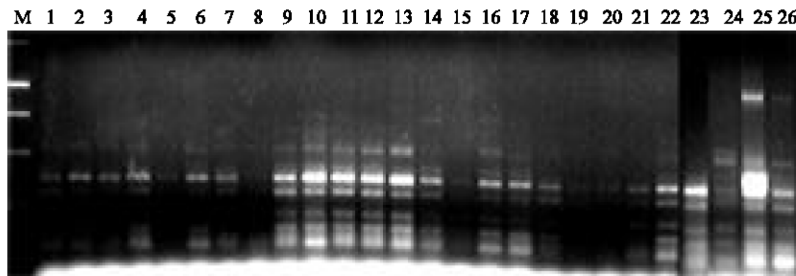

Fig. 4: The DNA fingerprinting patterns of *Orseolia* species using SCAR primer (OSSP16 forward: 5'TGATTACGCCCAGGTCGAT3'; OSSP1 reverse: 5'GATTACGCCCAG GTCACTGT3'), M: 1 kb molecular size marker

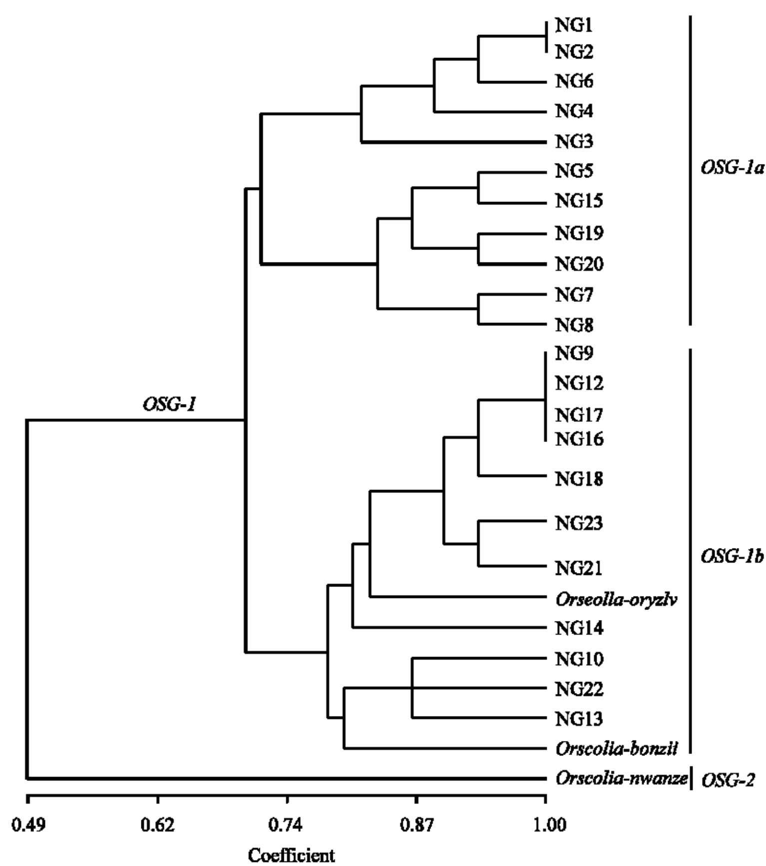

Fig. 5: Cluster analysis of *Orseolia* species as revealed by SCAR markers

Table 3: *Orseolia* species genotype distribution and population structure in Nigeria

Genotype	Subgroup	Occurrence (%)	*O. bonzii*	*O. nwanzei*	*O. oryzivora*
OSG-1	OSG-1a	42	-	-	-
	OSG-1b	54	+	-	+
OSG-2	-	4	-	+	-

-: Absent, +: Present

Using SCAR to determine genetic relationships should allow entomologists to identify and differentiate insect species before and after their release in the field. This will also assist scientists to study species composition existing in a locality before the introduction of a new species, thereby detecting the level of outcross between other species in the field (Maruthi *et al*., 2007; Armstrong *et al*., 1997; Behura *et al*., 1999). The present study confirms the earlier studies on the existence of *O. bonzii*, *O. nwanzei* and *O. oryzivora* in Nigeria (Nwilene *et al*., 2006), but *Orseolia* species genotype distribution and population structure in Nigeria as revealed by this study was not established in the previous study conducted by Nwilene *et al*. (2006). The distinct DNA fingerprints obtained in this study for different *Orseolia* species are potentially useful for their field diagnostic purposes to identify different biotypes of gall midge as well as detecting biotype variant outbreak in different locality (Maruthi *et al*., 2007; Behura *et al*., 1999). In addition, the DNA fingerprint defined by each *Orseolia* species should be useful for epidemiological surveys, identification of new species and to differentiate aggressive from non-aggressive species (Behura *et al*., 1999). This information will strongly assist breeding programmes aimed at effective development of cultivars with durable resistance to AfRGM in Nigeria.

CONCLUSION

Using SCAR-PCR analysis, identification and differential of *Orseolia* species in Nigeria has been made possible. Two major *Orseolia* species genotypes as well as *Orseolia* species genotype distribution and population structure in Nigeria were revealed by this study. The *OSG-1a* genotype that covers about 42% of yet unknown *Orseolia* species in Nigeria needs further investigation to establish their identity either as one new *Orseolia* species or as two or more new *Orseolia* species. This finding will further establish the actual number of *Orseolia* species in Nigeria, their genotype distribution and population structure towards achieving effective development of cultivars with durable resistance to AfRGM in Nigeria.

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