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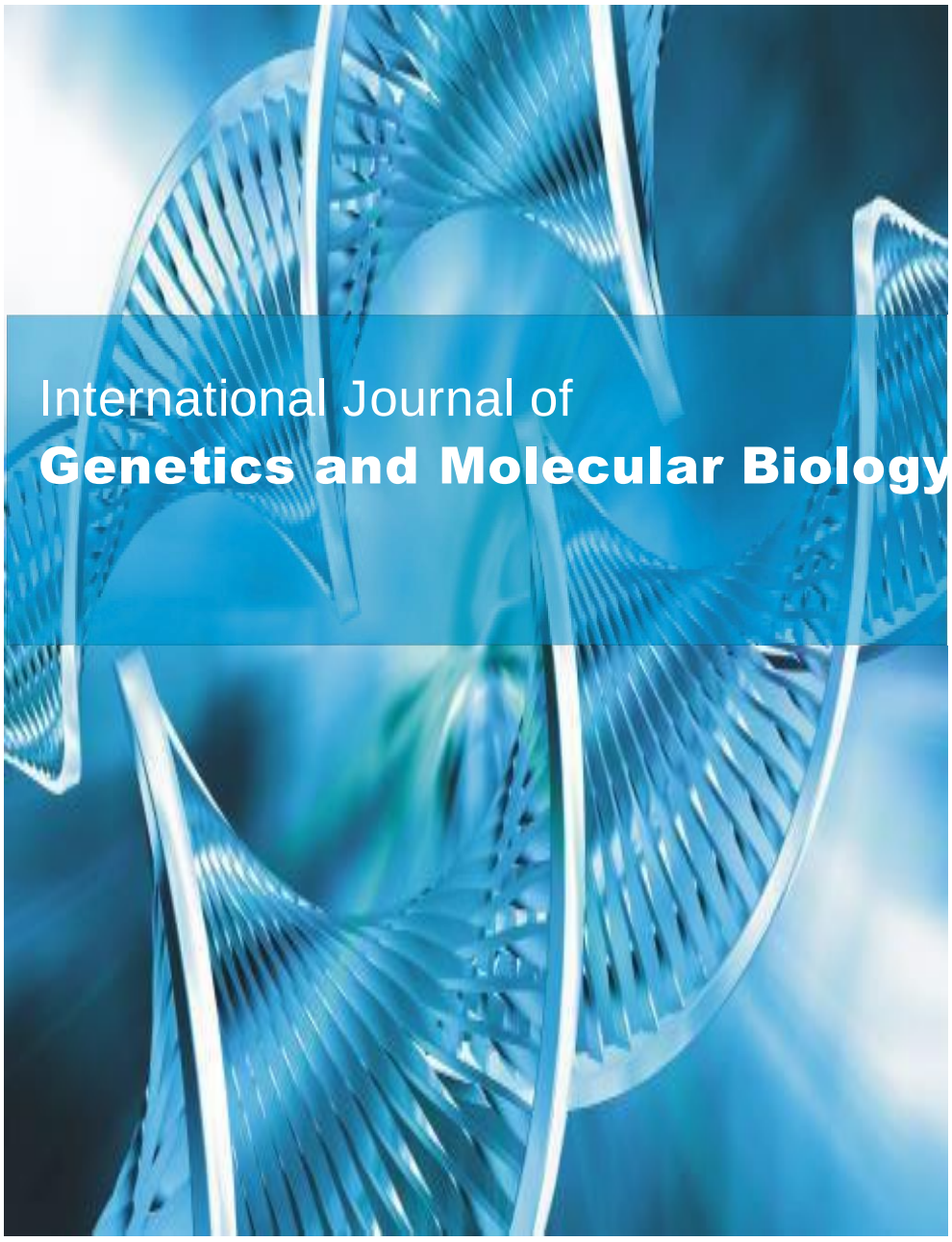

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Full Length Research Paper

Contribution of cytogenetic and molecular biology in disorders of sex development diagnosis: About 55 cases

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Disorders of sex development (DSD) when diagnosed early is important as it pose a real public health problem in Senegal. Among the supporting tools, molecular ones, which are not available everywhere are very useful. In this context, cytogenetic and molecular analyses were implemented in cytology laboratory at the Aristide Le Dantec hospital to enhance the DSDs diagnosis as well as evaluate the impact of the parents' age on such abnormalities. 55 cases of DSD have been received in the cytology laboratory for which cytogenetic (Barr chromatin and GTG karyotype) and molecular (SRY gene research) techniques have been used to characterize these anomalies according to the standards described in the international nomenclature. Three categories of DSD were found, namely 46,XX DSD, 46,XY DSD and chromosomal DSD. SRY is present in 4 patients 46,XX and absent in 3 patients 46,XY and results showed that the diagnosis is made earlier than previously (about 07 years). The study thus suggests the importance of complementarity (cytogenetics and molecular biology) in the diagnosis of DSD but also and especially the importance of early diagnosis from birth. Analysis of the epidemiological data also showed a slight correlation between maternal age and DSD. This showed us that a better characterization of DSD via increasingly powerful tools helps understanding on such pathologies and allows good medical care for patients.

Key words: Disorders of sex development (DSD), karyotype, SRY, hermaphroditis.

INTRODUCTION

Disorders of sex development (DSD) are rare abnormalities that can affect 1 to 3 of 10,000 children at birth (Bashamboo et al., 2010; Goultaiene et al., 2016; Mastrandrea et al., 2012). They are defined as individual

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whose genitals are difficult or even impossible to describe. However for Guillot (2008), these anomalies account for more than 10% of the population because any person who does not correspond to the morphological standards is *de facto* considered as an intersex. Moreover, these anomalies constitute an inadequacy between the sex reported at birth and the real sexual identity of the individual (Azonbakin et al., 2016; Gueniche et al., 2008; Guillot, 2008; Querfani et al., 2007). Advances in biology have shown us that the sex definition is not only based on physical criteria but requires an integrative approach (Hersmus et al., 2012). After the anatomical criterion (presence of penis or vagina), we have the gonadic (testicular or ovarian), genetic (XY or XX) or even the social criteria (male or female) (Azonbakin et al., 2016; Hersmus et al., 2012; Poulat et al., 1992; Sultan et al., 2001). An absence of one of these criteria can therefore lead to one of the known forms of DSD.

These anomalies were the subject of a new nomenclature based on an international consensus in 2006 (Diakité et al., 2013; Kim and Kim, 2012; Wiesemann et al., 2010). They can thus be classified as 46,XX DSD, 46,XY DSD and sex chromosome DSD, corresponding respectively to the terminology of female pseudo-hermaphroditism, male pseudo-hermaphroditism and true pseudo-hermaphroditism, which are now proscribed as they have a pejorative connotation (Lee et al., 2006). These DSDs cover broad clinical phenotypes that are essential to identify regardless the period of their expression (Folligan et al., 2012; Idrissi, 2012). Indeed, individuals affected by true hermaphroditism (also named ovotesticular DSD) possess both testicular and ovarian tissues associated with karyotypes that can be 46,XX (60% of cases), mosaics (30%) or 46,XY (only 10%) (Querfani et al., 2007). Furthermore, in sex chromosome DSD category we can find pathologies such as Turner and Klinefelter syndromes (Lux et al., 2009; Öçal, 2011).

On the other hand, the 46,XY DSD individuals derived from an inadequate masculinization of a genetically male embryo (Diakité et al., 2013; Goultaiene et al., 2016). They are associated with male gonads, but external genitalia remain ambiguous due to a pronounced deficiency of the hormone derived from testosterone (Azonbakin et al., 2016; Idrissi, 2012; Lee and Houk, 2008; Lin et al., 2007) . The last category 46,XX DSD refers to the presence of ovaries with external genitalia ambiguous and virilized to varying degrees such as penniform clitoris. This may be due to early exposure to androgens related to an adrenal tumor or inappropriate hormone therapy in pregnant women (Diakité et al., 2013; Folligan et al., 2012). The impact of these anomalies is heavy all over the world with a prevalence of 0.1 to 2% (Creighton and Minto, 2001). However, this estimate is even more worrying in the most disadvantaged areas because of the lack of opportunities to diagnose these infections as soon as possible (Azonbakin et al., 2016; Diakité et al., 2013; Folligan et al., 2012).

In Senegal, public cytogenetic structures specializing in the diagnosis and/or screening of congenital malformations are rare despite the rapidly expanding techniques (Matejka and Cribiu, 1987; Popescu, 1975; Popescu et al., 2000) widely used in cytogenetic to facilitate diagnosis in sub-Saharan Africa area (Diakité et al., 2013). Moreover, molecular biology techniques tend to be important in diagnosis of these diseases due to genetic recombination with, for example the SRY gene case that can sometimes be found on X chromosome (Faye et al., 2007; Gao et al., 2013). In addition, other techniques such as the study of Barr chromatin is sometimes used to make a first-line diagnosis of these anomalies (Artois and Salmon, 2009). This is a very rapid medical test to determine the percentage of Barr's corpuscles that correspond to the condensation of the second X chromosome in females, which range from 15 to 30% while it is between 0 and 5% for male epithelial cells. We can face a mosaic case where the percentage is found between 6 and 14% (Faye et al., 2007). Nevertheless, Barr's chromatin results can be influenced by several factors (Gueye et al., 2014) and the integration of these techniques could be of great value in the diagnosis of DSDs.

Despite the remarkable human variability, our gender identities are heavily constructed, socially and culturally and yet there is little data about the impact of these DSD in Senegal. Similarly, the genetic aspects of these abnormalities are little studied in Africa and therefore in Senegal especially in the most disadvantaged areas because of the lack of adequate diagnostic tools (Ediati et al., 2015). Due to the high birth rate and inbreeding but also to the continuation of procreation until a late age, every year there are a large number of births of children with genetic abnormalities, especially in families from disadvantaged areas (Juniarto et al., 2016). In addition, the management of these sexual disorders faces local beliefs that consider these abnormalities abnormal and shameful (Warne and Raza, 2008; Ediati et al., 2015), thus, leading to secrecy, social isolation and stigma (Ediati et al., 2017). These patients are therefore faced a real problem of identity, which makes difficult to see if the term is applicable to obtain a national incidence rate and therefore does not really reflect the importance of this problem. In this context, we aim to identify and classify through genetic analysis (cytogenetic and molecular techniques) the DSDs faced in Laboratory of Clinical Cytology - Cytogenetic - Reproductive Biology at the Le DANTEC hospital for better orientation and management of patients.

METHODOLOGY

Patients and samples

The study was carried out in the Cytogenetic Unit of the Clinical

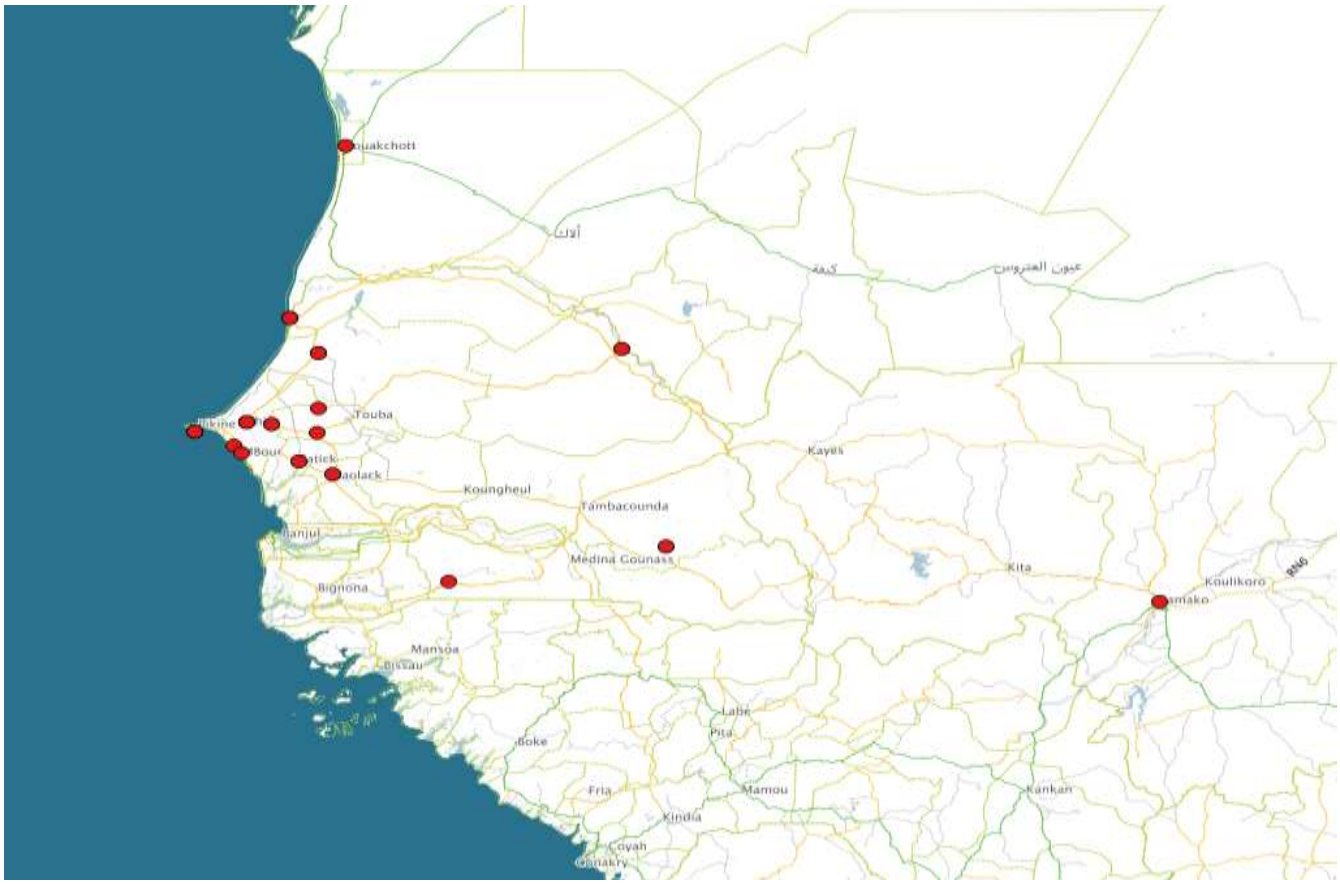

Figure 1. Geographic origin of patient. The red dot represents the origins of the patients.

Cytology-Cytogenetic-Reproductive Biology Laboratory of the Aristide Le Dantec Hospital (HALD), where only external patients received for a DSD indication have been integrated to the study. Clinical examination was performed and various information were taken such as declared sex (at birth), age of patient and parents at the diagnostic time, ethnicity and geographic origin as shown in Figure 1 (Table 1). All these information are then compiled and analysed under R v3.1.1. (R Development Core Team, 2008) using Fisher's exact test (with a significance level of 0.05) to see how the pathology is related or not to the age of parents.

Genetic studies

Several analyses were carried out, namely the Barr chromatin test, the GTG karyotype and the amplification of the *SRY* gene using three types of sampling. The specific techniques are explained in the following.

The chromatin test of Barr

This test was carried out from epithelial cells taken with a spatula by scratching the internal mucosa of the cheek followed by a spread on a slide and then an instantaneous fixing using the lacquer. Subsequently, cytoplasm lysis with chloric acid 1N (1N) was carried out at 56°C for 7 min followed by a series of hydration and dehydration with alcohol and distilled water. Finally, GUARD coloration was made before examining on an optic Microscope at least 200 interphasic nucleic (Faye et al., 2007).

GTG Karyotype

Venous blood was taken on a heparin tube and the cell culture was made within 72 h following the sampling according to the protocol described by Dia (2015), Gao et al. (2013) and Tijo and Levan (1956).

This technique was based on a culture at 37°C in the presence of 5% CO_2 and was done by inoculating 0.5 ml of blood. Before staining the Giemsa slides, enzymatic digestion was carried out in a trypsin solution for the labelling of G-band chromosomes followed by microscopic observation to analyse the metaphases and thus establish the GTG karyotype of the different patients concerned.
The establishment of the karyotypes was carried out using an imaging system composed of an epifluorescence microscope associated with the image capture and processing software "Leica CW 4000 cytogenetics".

Amplification of SRY gene

Blood was sampled on an EDTA tube and investigated the *SRY* gene using PCR amplification. *SRY* is a gene that plays an important role in masculinization because its codes for the factor that determining the testicles (TDF). This gene is located on Yp11.31 and can be transferred to the distal end of the short arm of the X chromosome or autosomes following an unequal crossing-

Table 1. Information of patients.

Patient							Parents		
ID patient	Age (months)	Reported sex	Region	Latitude	Longitude	Ethnic group	Age father	Age mother	Inbreeding
1	144	F	Dakar	14.6722222	-17.43166666666667	Peulh	-	-	oui
2	48	F	Saint-Louis	16.0333333	-16.5	Peulh	-	-	oui
3	204	M	Kaolack	14.1666667	-16.083333333333332	-	-	-	-
4	156	M	Dakar	14.6722222	-17.43166666666667	Bambara	62	52	non
5	180	F	Dakar	14.6722222	-17.43166666666667	Ouolof	-	40	oui
6	48	M	Dakar	14.6722222	-17.43166666666667	Socé	36	23	non
7	12	F	Dakar	14.6722222	-17.43166666666667	Toucouleur	48	40	oui
8	36	M	Dakar	14.6722222	-17.43166666666667	Ouolof	38	36	non
9	180	M	Fatick	14.3166667	-16.416666666666668	-	-	-	-
10	60	M	Saint-Louis	16.0333333	-16.5	-	49	45	oui
11	0.13	ND	Thiès	14.7905556	-16.924722222222222	Sérère	48	40	oui
12	444	M	Thiès	14.7905556	-16.924722222222222	-	76	63	non
13	7	M	Dakar	14.6722222	-17.43166666666667	Toucouleur	48	38	oui
14	24	F	Saint-Louis	16.0333333	-16.5	Toucouleur	46	40	non
15	36	M	Bamako	12.6333333	-7.983333333333333	Peulh	32	28	oui
16	24	F	Dakar	14.6722222	-17.43166666666667	Ouolof	43	37	non
17	0.6	ND	Kolda	12.8833333	-14.95	Peulh	50	34	non
18	72	F	Tambacounda	13.3	-12.816666666666666	Sarakholé	28	23	non
19	96	F	Dakar	14.6722222	-17.43166666666667	-	-	-	-
20	264	F	Dakar	14.6722222	-17.43166666666667	Peulh	69	57	non
21	0.33	F	Dakar	14.6722222	-17.43166666666667	Lébou	44	-	non
22	180	M	Dakar	14.6722222	-17.43166666666667	Sarakholé	-	46	oui
23	9	M	Kaolack	14.1666667	-16.083333333333332	Ouolof	-	24	oui
24	12	F	Tambacounda	13.3	-12.816666666666666	Socé	60	30	oui
25	216	F	Saint-Louis	16.0333333	-16.5	Peulh	40	35	oui
26	24	M	Thiès	14.7905556	-16.924722222222222	Ouolof	25	22	oui
27	1	ND	Dakar	14.6722222	-17.43166666666667	Lébou	55	35	non
28	24	M	Saint-Louis	16.0333333	-16.5	Ouolof	44	41	non
29	84	F	Dakar	14.6722222	-17.43166666666667	Mankagne	30	25	non
30	2	F	Fatick	14.3166667	-16.416666666666668	Ouolof	27	19	oui
31	12	M	Dakar	14.6722222	-17.43166666666667	Ouolof	32	24	oui
32	3	M	Thiès	14.7905556	-16.924722222222222	Peulh	26	20	oui
33	1	ND	Diourbel	14.655	-16.231388888888887	Ouolof	29	27	non

Table 1. Contd.

34	10	M	Dakar	14.6722222	-17.43166666666667	Peulh	46	33	non
35	10	M	Dakar	14.6722222	-17.43166666666667	Ouolof	28	27	non
36	60	M	Louga	15.6166667	-16.21666638888889	Maure	47	40	non
37	45	M	Thiès	14.7905556	-16.924722222222222	Sérère	30	24	non
38	18	M	Matam	15.6630556	-13.26096	Toucouleur	30	22	oui
39	36	M	Matam	15.6630556	-13.26096	Toucouleur	30	22	oui
40	276	F	Thiès	14.7905556	-16.924722222222222	Ouolof	-	-	oui
41	7	M	Dakar	14.6722222	-17.43166666666667	Ouolof	-	35	non
42	3	F	Nouakchott	18.1	-15.95	Toucouleur	-	-	oui
43	192	F	Thiès	14.7666667	-16.683333333333334	Ouolof	-	44	oui
44	24	M	Thiès	14.7666667	-16.683333333333334	Ouolof	-	44	oui
45	17	M	Thiès	14.5127778	-17.05	Sérère	-	33	oui
46	384	F	Thiès	14.7905556	-16.924722222222222	Sérère	50	47	non
47	2	F	Saint-Louis	16.0333333	-16.5	Ouolof	-	25	oui
48	240	F	Thiès	14.95	-16.216666666666665	Ouolof	66	53	oui
49	21	M	Louga	15.6166667	-16.21666638888889	Peulh	37	27	oui
50	72	M	Dakar	14.6722222	-17.43166666666667	Toucouleur	45	35	non
51	36	M	Louga	15.6166667	-16.21666638888889	Ouolof		30	oui
52	72	M	Dakar	14.6722222	-17.43166666666667	Lébou	50	44	non

F: Female; M: male; ND: not stated; "-": missing data.
Source: Authors

over during paternal meiosis (Barbaux et al., 1995; MacLean et al., 1997; Wu et al., 2014). Genomic DNA was extracted from 55 patients using the DNeasy 96 Blood & Tissue Kit (QIAGEN, Hilden, Germany). The *SRY* gene was amplified for a total of 22 patients selected who were carrying inconsistencies between karyotype and chromatin of Barr results but also ambiguous external genitalia. We used primers pair SRY-F 5-CAT GAA CGC ATT CAT CGT GTG GTC-3 and SRY-R5-CTG CGG GAA GCA AAC TGC AAT TCT T-3 (Settin et al., 2008). PCR reactions were performed in a 30 µL volume containing 17.1 µL of milliq water, 3 µL of 1X buffer, 0.6 µL of 0.5 mM $MgCl_2$, 0.1 µL dNTP, 3 µL of each primer at 1 µM and 0.1 µL of Taq (5 µL/µL). After an initial denaturation at 94°C for 2 min, conditions consisted of 35 cycles of a denaturation phase at 94°C/15 s, an hybridization at 65°C/20 s and elongation at 72°C/20 s. The program finished with a final elongation at 72°C/10 min.

RESULTS

Chromatin of Barr

Only two individuals (3.64%) of all patients did not perform the Barr chromatin test (Table 2). The three known categories have been found, these were the male (61.82%), the female (21.82%) and the intermediate chromatin sex (12.73%). For all the patients who did this test, 58% showed congruent results with the sex reported at the birth. However, for 42% of the patients, the diagnosis was different from the declared sex with seven possibilities that have been encountered (Table 3).

Indeed, following the Barr chromatin analysis, for the 22 patients whose sex was declared different from the chromatin sex results, three sex groups were proposed (Feminine, Male, and Undetermined). The first group "Feminine" concerns twelve individuals declared female, nine of them have a male chromatin sex, while the

Table 2. Genetic test results for each patient.

ID patient	Pathology category	Barr% chromatin	Karyotype	SRY
01	46,XXDSD	[15-30]	46,XX	Unused
02	46,XYDSD	[0-5]	46,XY	Present
03	46,XYDSD	[0-5]	46,XY	Unused
04	46,XXDSD	[15-30]	46,XX	Absent
05	46,XYDSD	[0-5]	46,XY	Unused
06	46,XXDSD	[15-30]	46,XX	Absent
07	46,XXDSD	[15-30]	46,XX	Unused
08	Chromosomal DSD	[6-14]	46,XY/46,XX	Absent
09	46,XYDSD	[0-5]	46,XY	Unused
10	46,XYDSD	[0-5]	46,XY	Unused
11	46,XYDSD	[0-5]	Unused	Unused
12	46,XYDSD	[0-5]	46,XY	Unused
13	46,XYDSD	[0-5]	46,XY	Present
14	46,XXDSD	[15-30]	46,XX	Unused
15	46,XYDSD	[0-5]	46,XY	Unused
16	46,XXDSD	[15-30]	46,XX	Absent
17	Chromosomal DSD	[6-14]	46,XX/46,XY	Unused
18	46,XXDSD	[6-14]	46,XX	Absent
19	46,XYDSD	[0-5]	Unused	Unused
20	46,XXDSD	[15-30]	46,XX	Unused
21	46,XXDSD	[6-14]	Unused	Unused
22	46,XYDSD	[0-5]	46,XY	Present
23	Chromosomal DSD	[0-5]	46,XX/46,XY	Unused
24	46,XXDSD	[0-5]	46,XX	Absent
25	46,XXDSD	[6-14]	46,XX	Present
26	46,XXDSD	[0-5]	46,XX	Present
27	46,XXDSD	[15-30]	46,XX	Absent
28	46,XYDSD	[0-5]	46,XY	Unused
29	46,XXDSD	[15-30]	Unused	Unused
30	46,XXDSD	Unused	46,XX	Absent
31	46,XYDSD	[0-5]	46,XY	Unused
32	46,XYDSD	[0-5]	46,XY	Unused
33	46,XXDSD	[0-5]	46,XX	Absent
34	46,XYDSD	[0-5]	46,XY	Unused
35	46,XYDSD	[0-5]	Unused	Unused
36	46,XYDSD	[0-5]	46,XY	Unused
37	46,XYDSD	[0-5]	46,XY	Unused
38	46,XYDSD	[0-5]	46,XY	Present
39	46,XYDSD	[0-5]	46,XY	Absent
40	46,XYDSD	[0-5]	46,XY	Absent
41	46,XYDSD	[0-5]	Unused	Unused
42	46,XYDSD	[0-5]	Unused	Unused
43	46,XYDSD	[0-5]	46,XY	Present
44	46,XYDSD	[0-5]	46,XY	Unused
45	Chromosomal DSD	[6-14]	46,XX/46,XY	Absent
46	Chromosomal DSD	[6-14]	46,XX/45,X	Unused
47	46,XXDSD	[0-5]	46,XX	Present
48	46,XXDSD	[0-5]	46,XX	Present
49	46,XYDSD	Unused	46,XY	Unused
50	46,XYDSD	[0-5]	46,XY	Unused
51	46,XYDSD	[0-5]	46,XY	absent

Table 2. Contd.

52	46,XYDSD	[0-5]	Unused	Unused
53	Chromosomal DSD	[15-30]	47,XXY	Unused
54	Chromosomal DSD	[15-30]	47,XXY	Unused
55	Chromosomal DSD	[15-30]	47,XXY	Unused

Source: Authors

Table 3. Differences between declared sex and chromatin sex.

Declared sex	Nuclear sex chromatin	Number of individuals
Female	Male	9
	Intermediate	3
Male	Female	5
	Intermediate	2
Undeterminate	Female	1
	Male	1
	Intermediate	1

Source: Authors

Table 4. Differences between declared sex and chromosomal sex.

Declared sex	Sex chromosomal	Number of individuals
Female	Male	4
Male	Female	3
	Mosaic	2
Undeterminate	Female	1
	Mosaic	1

Source: Authors

remaining three have an intermediate chromatin sex. The second group, "Male", consists of seven declared male patients, five of them had female chromatin and two intermediate chromatin sexes. The last group named "Indeterminate" referred to three patients of undetermined and/or undeclared sex at birth. In the latter group, we found the three known chromatin sex categories (Female, Male and Indeterminate).

The karyotype GTG

For the 55 patients studied, more than 85% performed karyotype tests. Five chromosomal formulas 46,XY (43.64%), 46,XX (29.09%), 46,XY / 46,XX (5.45%), 47,XXY (5,45%) and 46, XX / 45, X (1.82%) were found in the study population. After the analysis, 77% of the cases showed congruent results with the declared sex, while in 23% of the cases, the diagnoses were different with five possibilities encountered (Table 4). Indeed, in the "Feminine" group, we found four patients, all of them had a male chromosomal sex. In the group "Male" where we recorded five patients, three had a female chromosomal sex and 2 had a mosaic. In the last "Indeterminate" group, we obtained one female chromosomal sex patient and one chromosomal mosaic patient.

Search the SRY gene

The amplification of the *SRY* gene as shown in Figure 2 was based on the results of the karyotype and chromatin of Barr. For the 22 DNAs of patients thus analysed, *SRY*

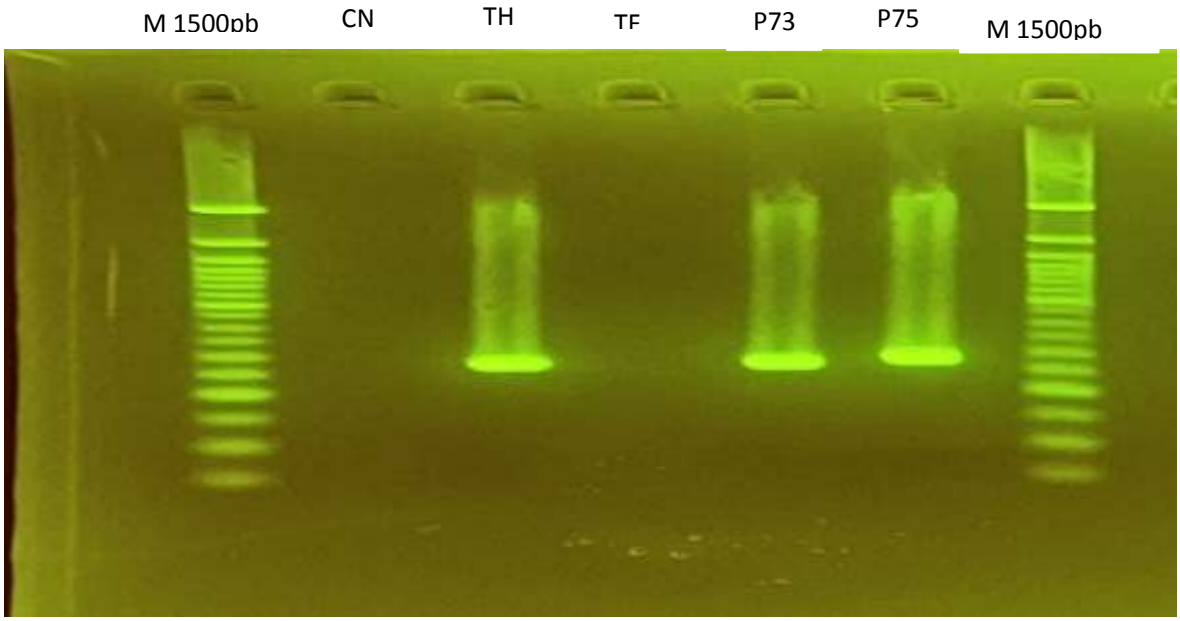

Figure 2. PCR amplification of a 254bp fragment from the SRY gene. M: 1500 bp size marker; CN: negative control no DNA; P: patient; TH: positive male control; TF: positive female control (no presence SRY).
Source: Authors

Table 5. Differences between chromosomal sex and chromatin sex.

Nuclear Sex Chromatin	Chromosomal formula	Number of individuals
Male	46, XX	5
	46, XY/ 46, XX	1
Female	47, XXY	3
Intermediate	46, XX	2
	46, XY	1
	46, XX/ 45, X	1

Source: Authors

was found (SRY+) in 9 patients of them while absent (SRY-) in the remaining (Table 2).

Comparisons between genetic data and DSDs classification

Results based on karyotype reveal that 72% are consistent with those of Barr chromatin and SRY gene search; that is to say, it refers to the same sex categories (Masculine or Feminine). On the other hand, for 28% of the cases the results are different from several cases of figures encountered (Table 5). Five patients showed an intermediate Barr chromatin level in the absence of the SRY gene and associated with a male (01), female (01) or mosaic (03) karyotype; other cases (08 patients) presented a male type of Barr chromatin associated with a karyotype of a female type (06 patients with the presence of the SRY gene in 03 of them), male (01 patient with no SRY) and mosaic (01). Finally, three patients had female type Barr chromatin levels associated with 47,XXY chromosomal formulas suggestive of Klinefelter syndrome.

The various pathologies listed are mainly in the anomalies of testicular development, anomalies of androgens and anomalies of ovarian development. The three classes defined in the international nomenclature, namely 46,XY DSD, 46,XX DSD and DSD chromosomes have all been found in our patients. Among these three classes, category 46,XY DSD is the most represented (p

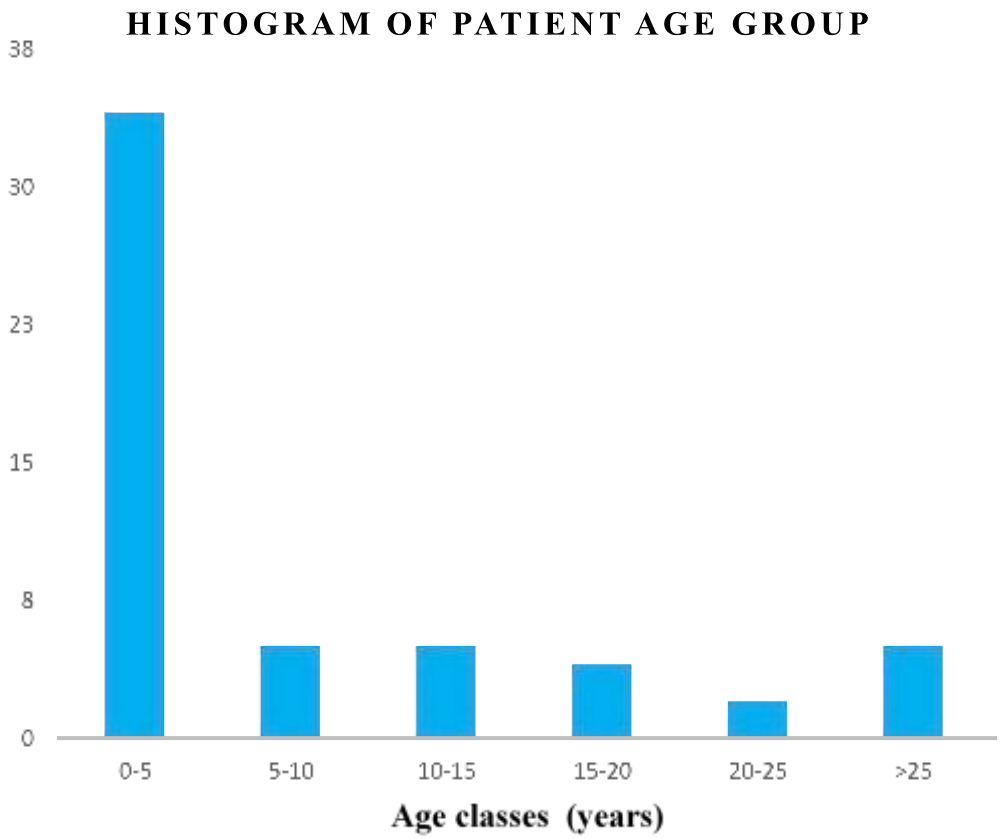

Figure 3. Histogram of the number of patients according to age class (ordinate; effective or percentage).
Source: Authors

= 0.0007). We also note in the context of chromosomal DSDs the presence of Turner syndromes in mosaic and Klinefelter as well as three cases of ovotesticular DSD.

Impact of age on pathology

The average age of patients received is 9.7 years with extremities ranking from 4 days to 48 years. The results of the different age classes are as shown in Figure 3. The most significant age group ($p = 1.239^{e-08}$) varies from 0 to 5 years with 67.28%, followed by the classes [6 to 10 years] and [11 to 15years] each representing 9.1%; followed by classes [16 to 20] with 7.28%; the class [>25 years] represented 5.45% and finally the class [21 to25 years] represented 1.82%. The Fisher test carried out between the age group and the pathology categories showed a very significant value ($p = 1.239^{e-08}$). The parental age ranged from 25 to 76 years for fathers with an average of 39.39 years and between 19 and 63 years for mothers with an average of 32.29 years. The correlation between the pathology observed in the patient and the age of the parents is slightly significant for mothers ($p = 0.02$) compared to fathers ($p = 0.08$).

DISCUSSION

Involvement of age in DSDs patients

The importance of DSD compared to other types of pathologies can be explained by the fact that these abnormalities most often affect sexual chromosomes with the presence in most of these patients of a phenotype suggestive organ (penoclitoris, ovotestis, testicular or clitoral hypertrophy, android morphotype, among others). As it is known, during ovogenesis, each of the 23 pairs of chromosomes had an equal risk of error during segregation, but these risks are higher in sexual chromosomes (Kamiguchi et al., 1994). Thus, the results obtained showed an age of consultation relatively young for these patients (almost 10 years). This average compared to those obtained in European countries seems to be considerably higher. Indeed, in those countries, 60% of the children with DSD are diagnosed at birth or even during the prenatal period (Gueye et al.,

2014; Mastrandrea et al., 2012). In our case, only 12 of the 55 cases of DSDs came for medical consultation before one year. However, it should be noted that the 3 oldest patients had Klinefelter's syndrome and therefore, came to consultation for primary sterility. The average age without the three later patients decreases to 7 years. This shows a great improvement of this average in Africa, if we just look back ten years ago in the studies carried out in the sub-Saharan area (an average: 14 years on the medical care of DSDs in Mali in 2003) (Kossi, 2003), 18.75 years on the sexual ambiguities in Dakar in 2001 (Ndiaye, 2001), 5 years concerning the surgical management of DSDs in Dantec Hospital HALD in 2004 (DIOUF, 2004). This delay in consultation (about 7 years) compared to developed countries may be due to the scarcity of specialized structures in these DSDs affections but also can be explained by several other reasons: lack of information and specific training that would lead to a rapid and early referral of patients for care (Folligan et al., 2012).

The socio-economic reasons are related to the fact that most of the patients are from the rural areas, which could cause inaccessibility to adequate services, the level of awareness but also the support (financial, logistic, etc). Furthermore, our data showed a slight correlation between maternal age (slightly increased) and the presence of these abnormalities in the patients studied.

Several studies have for long been interested in the impact of parental age on the occurrence of such pathologies. Maternal age is the only one that has an unequivocal link with number chromosomal abnormalities, especially trisomy 21 (Vekemans, 2003; Pellestor, 2004). On the other hand, and more recently, the advanced paternal age showed to be implicated in the occurrence of congenital anomalies due to the mutations that occur during spermatogenesis. Such mutations occurrence increases with age and should be checked in further studies later.

Contribution of genetic methods in the diagnosis of DSDs

Several chromosomal formulas have been found, highlighting both the importance of clinical diagnosis and the genetic methods used here (Barr Chromatin, Karyotype and SRY gene research). The results obtained by the karyotype, the Barr chromatin and the search for the SRY gene have allowed us to find a genetic sex congruent with the sex declared except for a few patients in whom the different analyses carried out one by one appear contradictory but interpreted together allow us to strengthen explanation of the phenotypes. Among these, three of them represented true cases of hermaphroditism corresponding to chromosomal DSDs. The presence of the two genotypes has the effect of diluting the percentage of Barr chromatin present in the patient, thus explaining the result obtained. Moreover, in four of the other patients we have evoked the translocation of the TDF on the X chromosome, which could be confirmed by molecular biology during analysis of SRY gene.

In the case of the few patients, the X chromosome (normally inactivated) had to be activated by the presence of this TDF, which allows it to behave like a Y chromosome and could therefore, explain the incongruence between the tests on the one hand, but also the presence of male external genitalia for these patients. Indeed, the SRY gene is often detected in 80% of XX men and 10% of true hermaphrodites XX (Barbaux et al., 1995) as shown in our study where the SRY gene was found only in individuals 46,XX. These different cases illustrated the fact that the Barr chromatin test must be done for any new born with an abnormality of the external genital organs but also must always be supplemented by a karyotype whenever possible to exactly know the chromosomal formula of the patient concerned (Ndiaye, 2001). Indeed, this examination already makes it possible to distinguish patients with more than one sexual chromosome X from those who have one or those who lack one. This does not mean that Barr chromatin is not useful when the karyotype has been performed as in some cases, Barr's chromatin may be indicative or even indispensable (Gueye et al., 2014).

The results also show that we can never be satisfied with Barr Chromatin alone in a DSDs diagnosis.

Cytogenetic analysis must always include a karyotypes (Diakité et al., 2013; DIOUF, 2004) which makes it possible to know the chromosomal formula of an individual (Ndiaye, 2001) and may prove to be important in the mosaics cases (46,XX/46,XY) as found in three of our patients. Other cases of mosaics have also been encountered, the latter being rather due to non-homogeneous syndromes and corresponding to the Turner's syndrome in our case (46, XX /45, X0) which dilute the barr chromatin thus found.

Finally, we found the Klinefelter syndrome in three of our patients whose Barr Chromatin tests revealed a rate that refers to the female chromatin sex, which is explained by the presence of the second X chromosome set found in the karyotype. On the other hand, the male phenotype is due to the expression of the genes on the Y chromosome. Indeed, the Y chromosome plays a dominant role in the determinism of the testis. Independently of the number of X chromosomes, an individual with only one Y chromosome develops in the male direction (Poulat et al., 1992; Barbaux et al., 1995; Al Jurayyan, 2011).

Conclusion

Disorders of sex development constitute a real public health problem and malformations are the leading cause of infant mortality. Cytogenetic is of great value in the

diagnosis and management of patients. The development and integration of the techniques of molecular biology via the research of the SRY thus made it possible to reinforce the reliability of the results. Indeed, the karyotype and the Barr chromatin have limits (intermediate level of chromatin of Barr or resolution of micro-rearrangements to be detected), hence the necessity to use molecular cytogenetics to refine the diagnosis. Analysis of epidemiological data showed a slight correlation between maternal age and pathology. Of course, these results could be related to the small size of our study population and the missing data encountered during the analysis. The latest studies have clearly shown that the age of consultation is becoming increasingly younger over the years, which is a major advance mainly due to the development of cytogenetic techniques but also and especially for molecular biology. This study induces us to orient ourselves towards molecular cytogenetics, which would allow many cases to find an answer and therefore a suitable treatment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

In silico analysis of mutations associated with genetic variability of the strain African cassava mosaic virus (ACMV) in three departments of Côte d'Ivoire

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Cassava (*Manihot esculenta* Crantz) is an important crop that constitutes staple food and income for 800 million people worldwide. Cassava yield in Côte d'Ivoire is reduced due to a variety of factors, including cassava mosaic disease. Despite the impact of the pathogen Cassava Mosaic Virus (CMV) on production, genetic diversity of this virus is rarely studied in Côte d'Ivoire. This study aims to assess the molecular variability of CMV occurring in three of large cassava production area of Côte d'Ivoire. Symptomatic and asymptomatic cassava leaves were collected for genomic DNA extraction and molecular identification was performed by polymerase chain reaction (PCR). Amplified DNA was sequenced and analyzed *in silico*. 68% of infections were identified as African Cassava Mosaic Virus strains. Sequences alignment to Genbank sequences showed high similarity with sequences of virus from Côte d'Ivoire, Ghana, Kenya, Cameroun, Madagascar, and Nigeria. The virus's rapid evolution was demonstrated by a high mutation rate at the protein level. A phylogenetic analysis also revealed seven new genotypes of ACMV strain. This result reflects a progressive genetic evolution of the virus strains, which could impact negatively on cassava crop in Côte d'Ivoire. This study proposed selecting resistant traditional cassava genotypes to control virus spread.

Key words: Cassava mosaic disease, ACMV, *in silico* analysis, mutation, resistant genotype, Côte d'Ivoire.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the largest staple food consumed by an estimated 800 million people worldwide (Alamu et al., 2019). It is grown almost everywhere in Côte d'Ivoire and is the country's second most important root crop after yam (Mobio et al., 2021). Although cassava has high agronomic potential, the fields are affected by pests and diseases, mainly Cassava Mosaic Disease (CMD), that hinders the productivity.

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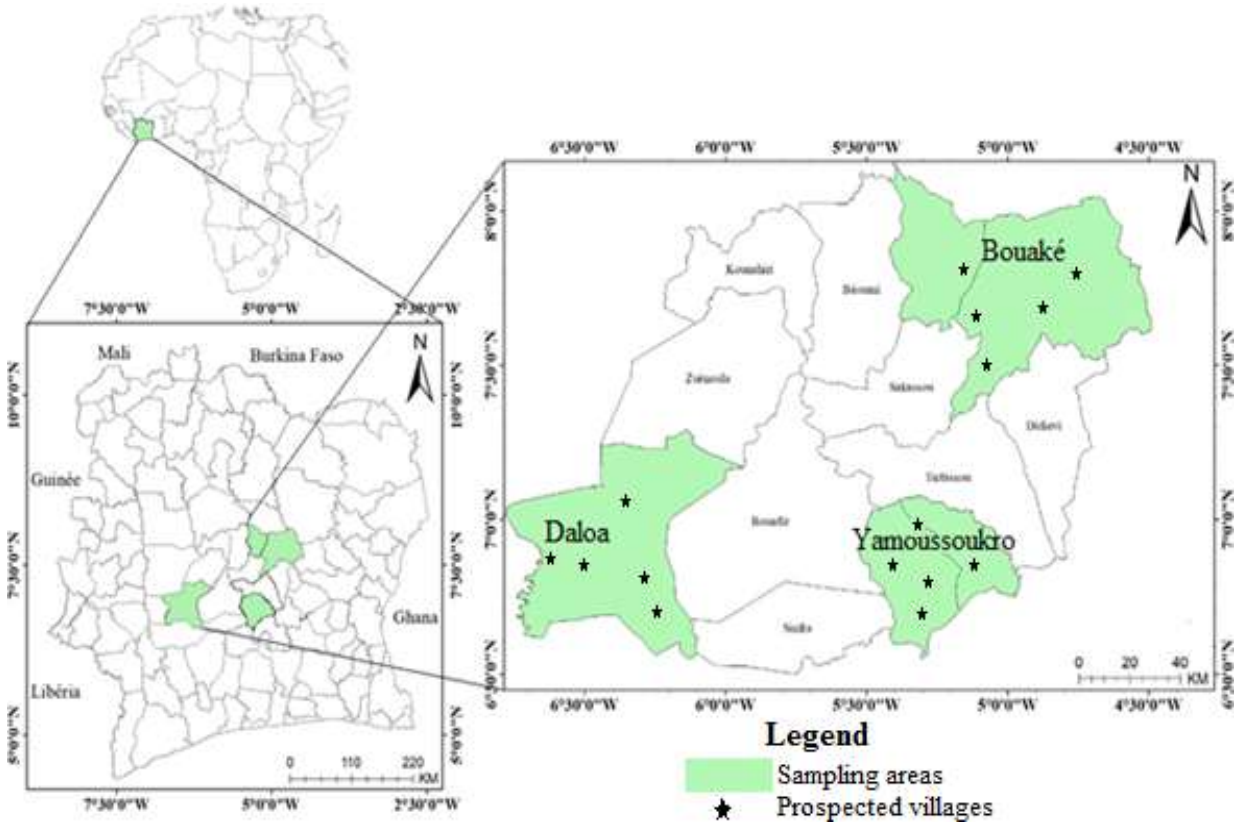

Figure 1. Geographic location of sampling sites.
Source: Authors

CMD is caused by Cassava Mosaic Geminiviruses (CMGs) which are transmitted by infected cuttings or whitefly *Bemisia tabaci* (De Bruyn et al., 2016). Cassava losses caused by Cassava Mosaic Viruses (CMV) can reach 90% (Yéo et al., 2020). Several strains of CMV have been identified in various countries and released in a public database (Elegba, 2018). Among these strains, nine have been described in Africa such as African cassava mosaic virus (ACMV), East African cassava mosaic Cameroon virus (EACMCV), East African cassava mosaic Kenya virus (EACMKV), East African cassava mosaic Malawi virus (EACMMV), East African cassava mosaic virus (EACMV), East African cassava mosaic Zanzibar virus (EACMZV), South African cassava mosaic virus (SACMV), African cassava mosaic Burkina Faso virus (ACMBFV), and Cassava mosaic Madagascar virus (CMMGV).

Two strains of the virus have been reported in Asia such as Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV). Among these viruses, studies conducted in Côte d'Ivoire have identified only two strains including ACMV and EACMV (Toualy et al., 2014). However, there is no information on genetic variants and intragenic mutations encoding these virus strains. Thus, the main objective of this study is to assess the molecular variability of ACMV strain in three of the large cassava production area of Côte d'Ivoire.

MATERIALS AND METHODS

Study area and sample collection

Survey for cassava leave sample collection were conducted from November, 2019 to August, 2020 in 191 small-holder farmer fields located in three of large cassava production departments of Côte d'Ivoire, from which 15 villages were selected according to the diversity of cassava varieties (Figure 1). These include N'Djebonoua, Diabo, Kongodekro, Kouakouyebouekro and Kekrekouakoukro villages from Bouaké department, Lolobo, Assanou, Oufouediékro, N'gbessou, and Akpessèkro villages from Yamoussoukro department, and Zakoua, Kibouo, Zaguiguia, Bribouo, and Zakaria from Daloa department. A total of 200 cassava symptomatic and asymptomatic leaves were collected and stored in labeled envelopes kept in a freezer at -80°C for subsequent DNA extraction.

Molecular characterization of virus strains

CMV genome amplification by PCR

Total nucleic acids were extracted from 2 g of each cassava leaf sample, as described by Yao et al. (2019). This extracted DNA was resuspended in 100 µl of elution buffer containing Tris-EDTA, and quality was tested on 1% agarose gel electrophoresis. DNA

solutions were stored at -20°C until amplification. PCR assays were performed to identify different virus strains using primers JSP001/JSP002 and ACMV/ACMV for African Cassava Mosaic Virus (ACMV) and JSP001/JSP003 for East African Cassava Mosaic virus (EACMV). PCR mix of each sample contained 5 µl of 10X buffer with $MgCl_2$ (Qiagen), 3.2 µL of deoxyribonucleoside triphosphates (dNTPs, 200 µM), 2.6 µL of each primer (10 pmol/µl, Eurogentec), 0.1 µL *Taq* polymerase (5 U/µL, Qiagen), 31.5 µL of pure water, and 5 µl of DNA. Reaction conditions were initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. After electrophoresis, PCR products were visualized by UV trans-illuminator. Known negative and positive control samples were included in all assays with 100 bp DNA Ladder to identify viruses according to allele sizes. The amplified products were scored (+) indicating the presence of the virus tested or (-) indicating the absence of the virus tested.

Sequencing of CMV strains

After amplification, 22 randomly selected CMV-positive PCR products were sent to Hong Kong BGI TECH SOLUTIONS for forward and reverse sequencing in ABI PRISM 3730 (Applied Biosystem) according to Sanger method (Sanger et al., 1977).

Bioinformatics and phylogenic analysis

The ACMV nucleotide sequences were compared to reference sequences available in Genbank genomic database of National Center for Biotechnology Information (NCBI) using the BLASTN local alignment search tool available online (http://www.ncbi.nih.gov). Protein sequences derived from nucleotide sequences were aligned and analyzed subsequently. These alignments were carried out in order to identify similarities between the CMGs variants obtained in this study and those from Genbank, as well as mutations between the aligned sequences. The Genbank sequence with the highest identity percent was chosen for each alignment. Finally, all sequences were compared using multiple alignments. Bioinformatic analyses were performed using the software Chromas Lite® 2.01 and Geneious prime 2021.1.1. CMV strain isolates were clustered based on their genetic relationships from phylogenetic trees using the Neighbour Joining (NJ) method with 1000 replicates. This analysis was performed using MEGA X software (Kumar et al., 2018). The evolutionary distances were generated by the Jukes-Cantor method based on the number of base substitutions.

RESULTS

Molecular detection and occurrence of the CMV strains

CMV amplification revealed the presence of ACMV and EACMV strains in the surveyed areas. Electrophoretic profile is characterized by DNA fragments of 783 and 1030 bp for ACMV and 780 bp for EACMV (Figure 2). Among 154 samples affected by CMD, 63.64% were due to ACMV while only 19.48 and 16.88% of these infections were caused by EACMV and coinfection ACMV/EACMV respectively, reflecting the predominance of ACMV strains.

Bioinformatic and phylogenetic analysis of ACMV sequences

Nucleotide sequence alignment

Nucleotide sequence analysis revealed genetic diversity in the ACMV strain which reflects the evolution of this strain in Côte d'Ivoire. Indeed, apart from a single sequence similar to a variant already identified in Côte d'Ivoire, isolated viruses were similar to variants already identified in five other countries available in the Genbank genomic database with identity percent between 96.5 and 98.9%. Out of the 22 sequences analyzed, 6 (27.27%) were identified to be homologous to the variant ACMV_GH:AK4A13 from Ghana with accession number MG250093, 5 (22.73%) sequences were similar to the variant ACMV_CM/YA under accession number AY211463 from Cameroun. Three sequences (13.64%) were similar to the Cameroun variant ACMV_CM/AK with accession number AY211461, and 2 (9.10%) were similar to the variant of Cameroun ACMV_CM/39 under accession number AY211462. Two others were comparable to the variant ACMV_CF:CF4AB from Madagascar, one of these sequences (4.54%) was similar to the variant ACMV_CF:CF72AB from Madagascar, one was identified to be similar to the variant ACMV_GH:FM14A from Kenya, one other was similar to the variant ACMV-[NG:So:03] from Nigeria and finally, one variant was similar to the variant from Côte d'Ivoire ACMV-[Ivory Coast] with accession number AF259894 (Table 1). All these variants were detected on the DNA-A of African cassava mosaic virus genome and encode the gene AV2 except two variants: ACMV_CM/YA and ACMV_CM/AK from Cameroon which encode the gene AV1.

Mutation's impacts on the evolution of virus variants

The nucleotide sequence alignments showed low mutation rates between 1.1 and 3.5%. ACMV_Dal1 nucleotide query sequence alignment with reference sequence of accession number MG250093 shows deletion (in green) at position 7 in the query sequence, transversions (in blue) at positions 115 (G115T), 304 (T304G), 640 (T640G) and 705 (G705T) and transitions (in yellow) at positions 147 (C147T), 190 (G190A), 209 (C209T), 276 (A276G), 328 (T328C), 367 (C367T), 646 (C646T) and 670 (C670T) (Figure 3). These mutations have resulted in nucleotide sequence modifications that have caused changes in amino acids codons and therefore of the protein sequences reflecting new variants (Figure 4).

Phylogenetic relationship

Based on the 99% clustering threshold, seven variants of

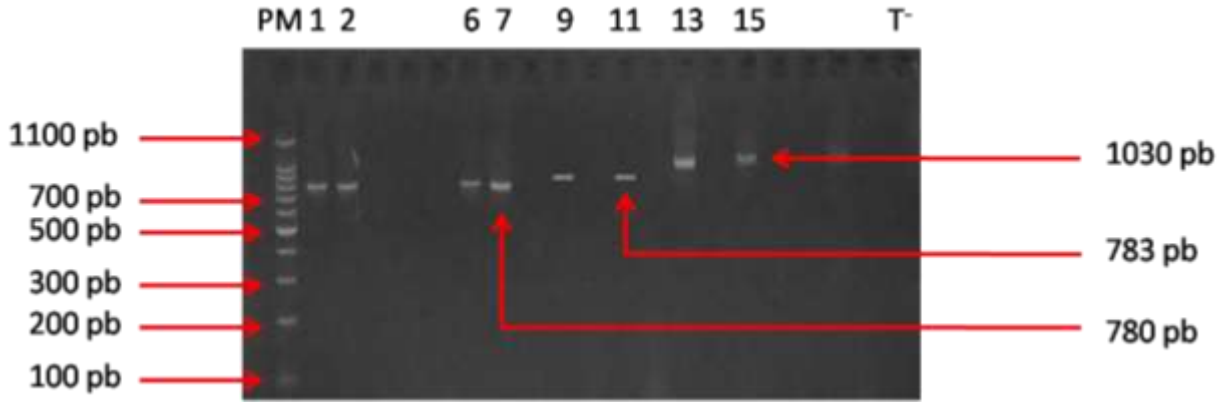

Figure 2. Electrophoretic profile on 2% agarose gel for cassava mosaic viruses' identification.
Source: Authors

Table 1. Characteristics of the Genbank sequences corresponding to the requested nucleotide sequences of ACMV strain.

Isolate	Variants	Accession No.	% Identity	Origin	Sequence length (bp)
ACMV-Bke1	ACMV CM/AK	AY211461	98.1	Cameroun	728
ACMV-Dal1	ACMV_GH:AK4A	MG250093	97.8	Ghana	719
ACMV- Dal2	ACMV_ CF:CF4AB	KJ887756	98.6	Madagascar	730
ACMV- Dal3	ACMV_CF:CF4AB	KJ887756	98.5	Madagascar	723
ACMV-Yak1	ACMV_CF:CF72AB	KJ887780	98,6	Madagascar	708
ACMV-Dal4	ACMV CM/AK	AY211461	98.1	Cameroun	728
ACMV_Bke2	ACMV_GH:AK4A:13	MG250093	98.7	Ghana	720
ACMV_Yak2	ACMV-[NG:So:03]	EU685322	98.3	Nigeria	708
ACMV_Dal5	ACMV_CM/YA	AY211463	97.3	Cameroun	708
ACMV_Yak3	ACMV_GH:AK4A	MG250093	98.9	Ghana	708
ACMV-Yak4	ACMV_ CM/AK	AY211461	98.3	Cameroun	708
ACMV-Dal6	ACMV_ CM/39	AY211462	98.4	Cameroun	741
ACMV_Yak5	ACMV_GH:AK4A	MG250093	98.3	Ghana	708
ACMV_Bke3	ACMV_GH:FM14A	MG250159	98.3	Kenya	708
ACMV_Dal7	ACMV_CM/YA	AY211463	97.3	Cameroun	708
ACMV_Bke4	ACMV_GH:AK4A:13	MG250093	98.4	Ghana	749
ACMV_Yak6	ACMV_GH:AK4A:13	MG250093	98.6	Ghana	732
ACMV_Bke5	ACMV-[Ivory Coast]	AF259894	96.5	Côte d'Ivoire	707
ACMV_ Bke6	ACMV_CM/YA	AY211463	98,3	Cameroun	719
ACMV_ Bke7	ACMV_CM/YA	AY211463	98.3	Cameroun	708
ACMV_ Dal8	ACMV_CM/YA	AY211463	97.3	Cameroun	707
ACMV_ Yak7	ACMV_ CM/39	AY211462	98.4	Cameroun	741

Source: Authors

the ACMV strain could be defined from the 22 isolates studied. Three clusters and four single ACMV isolates protein sequences were identified regarding the phylogenetic tree. While cluster 1 comprises isolates from all the departments surveyed, clusters 2 and 3 included only isolates from the departments of Daloa and Bouaké, respectively (Figure 5). Three out of the four single ACMV isolates were from Daloa department and one from Bouaké. All isolates from Yamoussoukro department are in cluster 1.

DISCUSSION

The study showed the predominant of ACMV strain among Cassava Mosaic Viruses in Côte d'Ivoire. This result is in agreement with Toualy et al. (2014). This virus has been shown to be the predominant virus in several

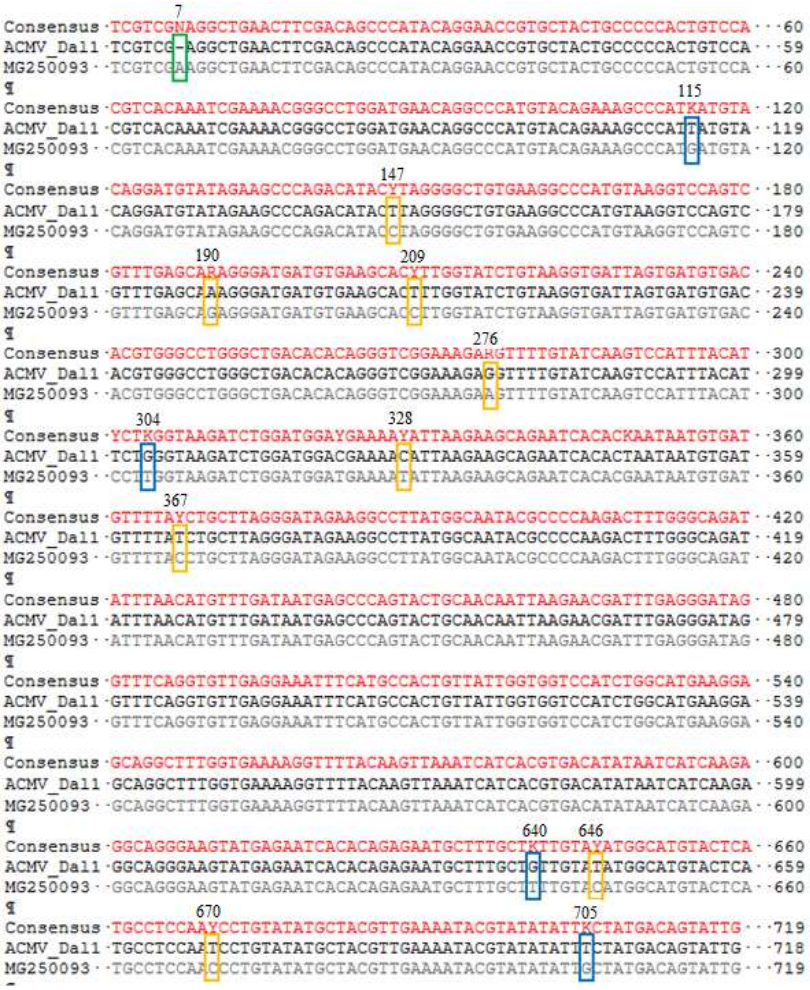

Figure 3. Alignment of nucleotide sequence of ACMV_Dal1 (in black) on the reference sequence of accession number MG250093 (in grey).
Source: Authors

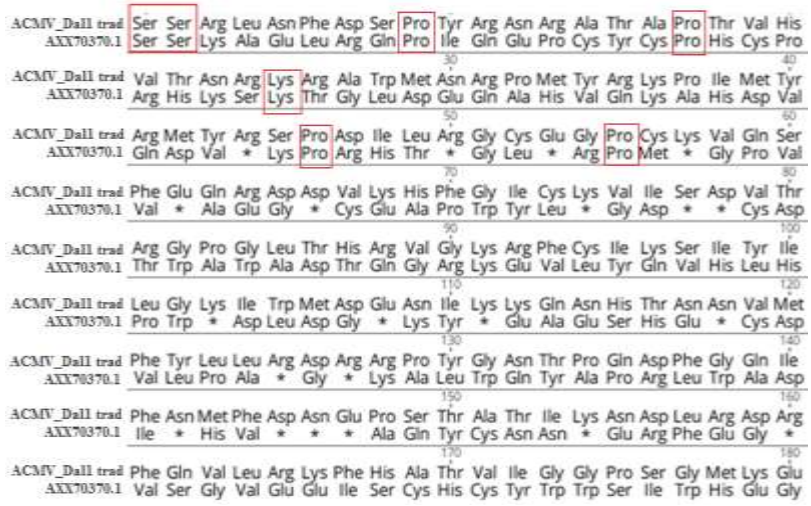

Figure 4. Mutations observed by alignment of the protein query sequence of ACMV_Dal1 with its reference sequence of accession number AXX70370.1.
Source: Authors

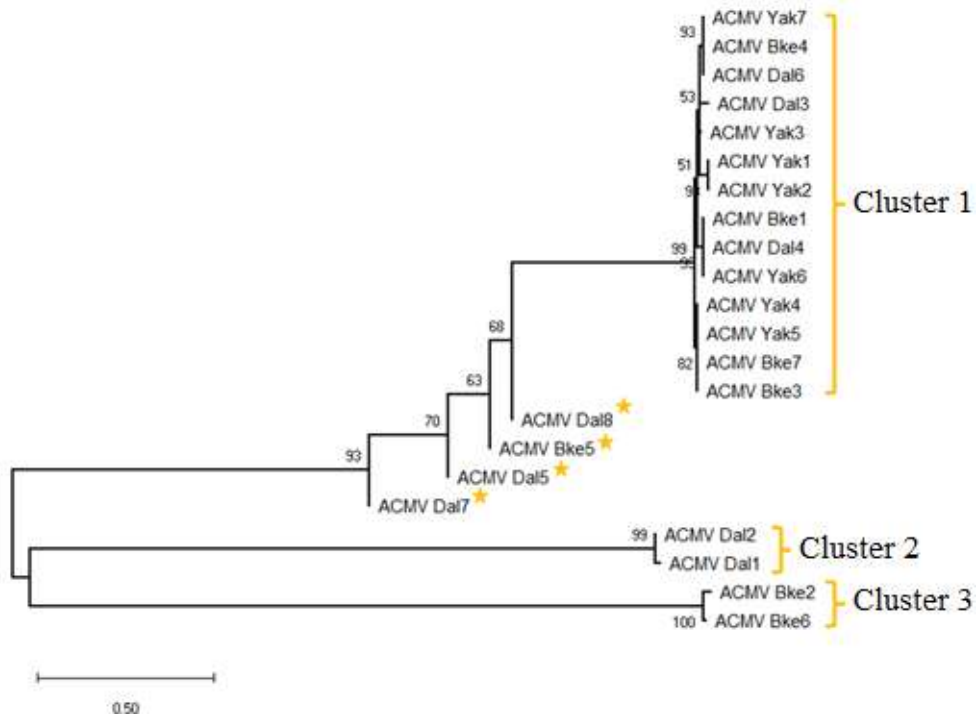

Figure 5. Cladistic structure of the protein sequences of ACMV isolates according to the neighbour joining tree.
Source: Authors

other countries where CMD occur such us Burkina-faso and Madagascar (Tiendrébéogo et al., 2012; Harimalala et al., 2012). The results of this study revealed an important genetic diversity within ACMV strain. The variants isolated using 22 full-length nucleotide sequences of ACMV from farmers' fields were similar to variants of six African countries including Côte d'Ivoire according to the Genbank genomic database. These are variants ACMV_GH:FM14A from Kenya, ACMV_GH: AK4A:13 from Ghana, ACMV_ CF:CF4AB and ACMV_CF:CF72AB from Madagascar, ACMV_CM/YA and ACMV_CM/AK; ACMV_CM/39 from Cameroun, ACMV-[NG:So:03] from Nigeria and ACMV-[Ivory Coast] from Côte d'Ivoire with similarity percentage between 96.5 and 98.9%. These high similarities may indicate that viruses originate from these different countries. The low mutations detected in the nucleotide sequences indicate that isolates are derived from existing variants from these countries with some mutations due to environmental. This result is supported by Mulenga et al. (2016) who showed that CMV diversity in Zambia is caused by cuttings exchange with other countries. Also, cutting, which is the main means of cassava production, has an important role in the spread of viruses. This is the main factor of CMD development (Harimalala et al., 2015). In fact, anthropogenic activities such as exchange of planting material has played a major role in the spread of CMGs outside of their previously reported geographic ranges, facilitating the colonization of new niches (Legg et al., 2014). Missing awareness of the farmers to the risk posed by uncertified plant material, the difficult access to virus-free plant material, and the preference of some varieties by some farmers are among reasons of CMD propagation in addition to whiteflies *Bemisia tabaci* contribution (Legg et al., 2015).

According to Crossley and Snyder (2020), the insects *B. tabaci* provide long-distance flights that can carry them from one area to another. Moreover, underlying mechanisms such as mutation have been reported to play a role in the evolution of geminiviruses (Ramesh et al., 2017). In this study, although there were very few mutations in the nucleotide sequences, they favored the evolution of the viruses which is reflected in the very high mutations in protein sequences. According to Elegba (2018), mutations constitute the diversification engine of viruses because Geminiviruses are single stranded DNA viruses that replicate quickly with proofreading and mismatch repair capacity. These processes strongly help virus acquiring great genetic variability and thus creating new arrangements within the genome (Lefeuvre and Moriones, 2015). Thus, mutant gradually becomes a new virus that is often more dangerous than the initial one. When a mutation in a coding region results in an amino acid change, it can be deleterious to its host plants.

Phylogenetic analysis realized using the protein sequences of the 22 ACMV isolates revealed three

clusters and four singles, reflecting new variants of this strain and high genetic diversity of the virus in Côte d'Ivoire. However, Asare et al. (2014) contend that the viruses' high genetic variability may contribute to the new development of CMD and have serious implications for production. The genetic variability of the strain ACMV observed in this study represents an ideal condition for emergence of others severe variants through numerous possibilities of intra- or intergenic recombination and presents a major epidemiological risk for cassava crop (Elegba, 2018). As a result, this study should be able to challenge all relevant actors regarding CMD control measures. Cassava cultivars that are virus-resistant may be the most effective control measure (Elegba et al., 2020; Houngue et al., 2019).

Conclusion

Molecular genetics and *in sillico* analyses on Cassava Mosaic Virus highlights its perfect evolution in Côte d'Ivoire, with several variants identified. These variants are highly similar to some variants discovered in African countries such as Ghana, Kenya, Cameroon, Madagascar, Nigeria, and Côte d'Ivoire. Genetic variations are reflected by various mutations observed involving natural selection, human activities, and environmental factors. Seven ACMV genotypes represented by three genetic clusters and four single isolates were identified and could be considered as new variants of CMV in this study. As a result, it appears critical to seek cassava cultivars that are resistant to Cassava Mosaic Viruses for effective control.

As a precaution, genomic sequencing of the other mosaic virus strains should be performed in order to identify the different variants and their distribution in Côte d'Ivoire for effective control. Finally, traditional mosaic-resistant varieties would need to be identified for the proposed control methods.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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