International Journal of Botany

ISSN: 1811-9700

science alert

ANSInet
an open access publisher
http://ansinet.com

International Journal of Botany 2 (2): 187-192, 2006
ISSN 1811-9700

Cytology and Molecular Cytogenetics of *Cucumeropsis mannii* Naudin: Implications for Breeding and Germplasm Characterization

[1]Julian O. Osuji, [1]Bosa E. Okoli and [2]J.S. Pat Heslop-Harrison
[1]Department of Plant Science and Biotechnology and
Regional Centre for Bioresources and Biotechnological Research,
University of Port Harcourt, P.M.B. 5323, Port Harcourt, Nigeria
[2]Department of Biology, University of Leicester, LE1 7RH, UK

Abstract: Cytological and molecular cytogenetic studies were conducted on the two accessions of *Cucumeropsis mannii* Naudin to establish the true somatic chromosome count, investigate the occurrence and number of ribosomal RNA genes in its genome as well as investigate any cytological and cytogenetic variation in the two cultivars of the monospecific taxon. Chromosomal counts from squashed root tips stained with FLP orcein and DAPI stained mitotic chromosomes showed a somatic count 2n = 24 for this species. The chromosomes were small in size measuring 3-4.5 μm long and mostly varied from submetacentric to subacrocentric in shape. Both 18S-5.8S-25S and 5S rRNA genes were observed in the genome. Four sites, comprising two major and two minor, of 18S-5.8S-25S rRNA genes were present on four of the largest chromosomes (4.5 μm long) while two sites of the 5S rRNA genes were observed on two other individuals of the largest chromosomes. This report establishes the correct somatic chromosome count as well as the ribosomal rRNA features of this taxon. It also shows no cytological variation between the two cultivars of this species.

Key words: *Cucumeropsis mannii* Naudin, cultivars, somatic chromosome number, chromosome size, ribosomal RNA genes

INTRODUCTION

Cucumeropsis mannii Naudin (Annales des Sciences Naturelles; Botanique, Sér. 5,5: 1866; Synonym: *Cucumeropsis edulis* (Hooker fil.) Cogn., Basionym: *Cladosicyos edulis* Hook. f.) is a monoecious and monospecific taxon in the Cucurbitaceae (Order: Violales and Subclass: Dillenidae). It originates and is endemic to West and Central Africa (Okoli, 1984). Being partially drought-resistant, cultivation of *C. mannii* is mostly around the tropical rain forest regions of West and East Africa. The plant is flagelliflorous and is often cultivated alongside *Dioscorea* spp. (yam), *Abelmoschus esculentus* (L.) Moench. (okro) and *Telfairia occidentalis* Hooker fil. (fluted pumpkin). The seeds are usually planted close to small trees, shrubs, fences or other similar support, which act as stakes for it.

In regions where it occurs, *C. mannii* is of great economic importance (Zoro Bi *et al*., 2003; Andres, 2004). It serves mainly as food; the most regularly used part of it being the seed. The seeds (milky in colour) can be eaten roasted. Otherwise, the testa can be removed and the oily white proteinous cotyledons are gathered, pulverized and used as a native soup thickener. This species is nutritious (Okigbo, 1975) and also medicinally important (Gill, 1992; Sofowora, 1986). The cultivar, which has oblong-rounded fruit with more robust seeds, appears more favoured as a delicacy than the one with more cylindrical fruit with slimmer seeds. Rodents more readily savour the fruit and seeds of the oblong-rounded fruit type than the cylindrical type. The fruits and seeds are boiled and eaten along with yam (*Dioscorea* spp.) while decoction of the leaves are used as mild laxative and contains steroids, tryptophans, vitamin C and carbohydrates (Gbile, 1986).

Though very important, this domesticated species has attracted only little attention in research and crop improvement. Hence little is known about its scientific prospects. For example, not much is known about the cytology and cytogenetics of this taxon. Though cytological work in the Cucurbitaceae is reportedly difficult (Whitaker, 1974; Jeffrey, 1980), a count, 2n = 2x = 22, was reported for *C. mannii*. King

Corresponding Author: Julian O. Osuji, Department of Plant Science and Biotechnology, Regional Centre for Bioresources and Biotechnological Research, University of Port Harcourt, P.M.B. 5323, Port Harcourt, Nigeria Tel: 234 0 803 3404117, 234 0084 775304

(1974) opined that the cell chemistry of Cucurbitaceae is different from other plant taxa. Poor stainability and small chromosome size combined with the absence of a standard nomenclature of genes did not encourage cytogenetic investigations in the Cucurbitaceae (Roy *et al*., 1991). However, occurrence of fairly large chromosomes has been reported for some species, in particular *Cucumis* spp., which is amenable to detailed karyotypic investigations (Ramchandran *et al*., 1983). But the chromosomes in most genera are relatively small (Okoli, 1984).

Beyond routine chromosomal characterization, molecular characterization of plant and animal species has contributed much desirable information required for their characterization, exploitation, conservation and improvement. Hence, occurrence and distribution of ribosomal RNA genes (i.e., rDNA) has been investigated and reported in many species. The ribosomal RNA gene has been widely investigated in animals, including the Diptera, *Glossina* (i.e., tsetse fly, Willhoeft, 1997) and a wide variety of wild and cultivated plants. The number of sites, pattern of distribution, strength of signal and other attributes of rDNA has been used to characterize, classify and discriminate between species as well as show taxonomic relationships and to explain phylogeny of hybrids, cultivars, species and genera (Osuji *et al*., 1998). To date, rDNA studies have not been conducted in the Cucurbitaceae.

This species has been quite neglected and currently faces the threat of extinction. Considering its ethnobotanic value and genetic endowments, it is important to conduct proper genomic characterization of the *C. mannii* germplasm. Data so-generated would help to improve its utility and enhance exploitation of its genepool as a ready source of novel genes for both intra-and interspecific improvement of related economically viable species. Hence, the aim of this study was to establish the correct chromosome number and to investigate the occurrence and number of sites of the two major ribosomal RNA genes in this species.

MATERIALS AND METHODS

The plant materials used for this research were gathered from farms in different parts of south eastern Nigeria. The accessions were established in the Botanic garden at the Regional Center for Bioresources and Biotechnological Research, University of Port Harcourt, where they were maintained for over ten years. Healthy roots, about 10 mm long were generated by plating seeds, previously sterilized with 30% chloral solution, on wet filter paper in a dark cabinet and by germinating seeds *in vivo*.

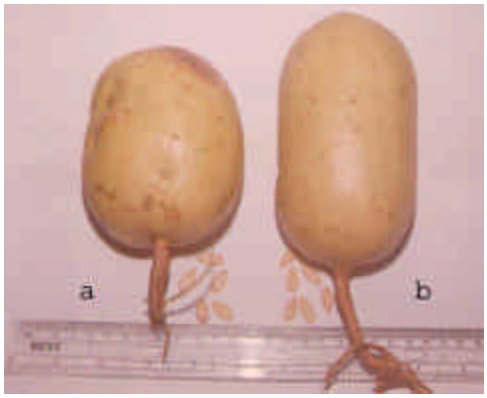

Fig. 1: Mature fruits of *C. mannii* showing structural variation between the fruits and seeds of the two cultivars. One cultivar has oblong-round fruits with slightly larger seeds (a) while the other has cylindrical fruit with longish and narrow seeds (b)

Cytological studies: Mature fruits were obtained from plants representing the two varieties of *C. mannii*. Fresh viable seeds were extracted from mature fruits (Fig. 1), air-dried for two weeks and germinated to generate fresh healthy roots. Root tips were pretreated in 2 mM 8-hydroxyquinoline for 3 h and fixed in 3:1 v/v ethanol acetic acid for 24 h. Root tips were then stored in 70% ethanol prior to acid hydrolysis with 5% (2 min) and 9% (3 min) HCl followed by squashing in FLP orcein (Osuji, 2003). Mitotic slides were observed under Leitz Diaplan microscope.

Sites of ribosomal RNA genes: The method used for chromosome spreading followed Osuji *et al*. (1997, 1998). Fresh primary roots were harvested from chloral-sterilized seeds germinated by plating on moist filter papers. The roots were pretreated in 2 mM 8-hydroxyquinoline for 3 h and fixed in 3:1 ethanol acetic acid for 12 h. Fixed root tips were stored in 70% ethanol prior to hydrolysis. Roots were washed thrice in 1×enzyme buffer (0.01 M citric acid-sodium citrate, pH 4.6) for 9 min with shaking to remove fixative and preservative. Tips measuring about 4-5 mm were excised and hydrolyzed in 1.5 mL enzyme mix (2% cellulose, Onuzuka R10, Yakult Honsha Co., Tokyo and 20% liquid pectinase, from *Aspergillus niger*, Sigma) in enzyme buffer (Osuji *et al*., 1997; Schwarzacher *et al*., 1989) for 1 h at 37°C.

Digested root tips were washed thrice with 1 × enzyme buffer and squashed in a drop of 60% glacial acetic acid under No. 1 (18×18 mm) coverslip. About 1 mm of the actual tip of the root was squashed in a drop of 60% glacial acetic acid. The slides were flattened out each

with a firm thumb pressure. The coverslip was removed after freezing on dry ice and the slides were air-dried.

On each slide, the area containing the chromosome spread was treated with 100 μL of 100 μg mL^{-1} DNase-free RNase in 2×SSC (20×SSC, 3 M sodium chloride plus 0.3 M trisodium citrate) solution. The slide was covered with a plastic coverslip and incubated at 37°C for 1 h. Slides were washed thrice, each for 5 min, in 2×SSC (on a shaker) and treated with 10 μg mL^{-1} pepsin, covered with a plastic coverslip, incubated at 37°C for 30 min and washed for 5 min in 2×SSC (shaking). Washed slides were pre-fixed with 4% paraformaldehyde twice each for 5 min, washed twice in 2×SSC (for 5 min each), dehydrated in 70% alcohol for 5 min and air-dried.

One of the two probes used was pTa71, isolated from *Triticum aestivum* (Gerlach and Bedbrook, 1979). It has a 9 kb EcoRI fragment containing 18S-5.8S-25S rRNA genes and intergenic spacer segments. The other probe was pTa 794 with a complete 410 bp 5S rRNA gene unit and spacer region also isolated from *T. aestivum* (Gerlach and Dyer, 1980). The probe pTa71 was labeled with biotin-11-dUTP (Boehringer Mannheim) while pTa794 was labeled with digoxigenin-11-dUTP (Boehringer Mannheim). The hybridization mixture comprised 20-30 ng of probe DNA (0.5 μL pTa71 and 1 μL pTa794), 15 μL 100% formamide, 6 μL 50% dextran sulphate, 10% sodium dodecyl sulphate, in water), 2 μg salmon sperm DNA, 3 μL 20×SSC in a total volume of 30 μL probe mixture per slide. Denaturation of the mixture was at 70°C for 10 min in a water bath and cooling was for 5 min in ice. Chromosome preparations were covered with the hybridization mixture and then plastic coverslips and denatured at 80°C for 5 min in an Omnislide humid chamber. The preparations were then incubated at 37°C for 12 h.

Preparations were washed after hybridization (for 5 min each time) twice with 2×SSC, twice with 20% formamide in 0.1×SSC (very stringent wash to allow sequences more than 85-90% homology to remain hybridized) and twice in 2×SSC. All the washing was at 42°C on a shaker. The final washing with 2×SSC was also twice, each for 5 min. This was followed by washing with 4×SSC/0.02% Tween 20 (detection buffer) on a shaker at room temperature.

In order to detect hybridization sites, preparations were first blocked by incubating with 100 μL per slide of 5% w/v bovine serum albumen (BSA) in 4×SSC/0.02% Tween 20, covered with plastic coverslips at room temperature for 5 min. Hybridization sites were detected by adding 100 μL of detection mixture per slide, which contained Cy3-streptavidin and sheep anti-digoxigenin conjugated to fluorescein isothiocyanate (anti-dig-FITC) in BSA and incubated for 1 h at 37°C. The chromosome preparations were washed twice, each for 5 min, in 4×SSC/Tween 20 and counterstained with 6 μg mL^{-1} 4'-6-diamidino-2-phenylindole (DAPI) in McIlvaine's citrate buffer (pH 7.0) solution under plastic cover slips for 10 min. Counterstained preparations were quickly washed in 4×SSC/Tween 20 and mounted in Antifade solution (Citifluor Glycerol/PBS solution AF1) for observation under Leitz epifluorescence UV microscope with Leitz filter sets (A for DAPI, 12/3 for Cy3, N2 for FITC and Omega Triple Bandpass). Photomicrographs were taken from good slides.

RESULTS

It was difficult to obtain good slides with clearly stained chromosomes from conventionally made cytological slides stained with FLP orcein. Low number of metaphase yield was observed in most of the slides. Instead, late prophase cells with uncondensed ends occurred more frequently (Fig. 2). The chromosomes were relatively small in size measuring about 3-4.5 μm long. A diploid chromosome number of 2n = 24 was counted for both cultivars of this taxon (Fig. 2a and b). There was slight cytoplasmic staining of mitotic tissue, which was deeper when lower strength of HCl was used to hydrolyze the root tips or when hydrolysis was abridged. Figure 2a shows non-uniform condensation of mitotic chromosomes. Spiralisation of the chromosomes tended to start from the centromere and extends to the telomeres of the chromosomes. A majority of the chromosomes were submetacentric and subacrocentric in structure. There was no observable cytological difference between the two cultivars.

The 18S-5.8S-25S rRNA gene: *In situ* hybridization showed the presence of 18S-5.8S-25S rRNA genes. Four chromosomes carrying signals representing sites of this gene were observed. The four signals comprised two major and two minor sites of the ribosomal gene. The chromosomes, which carried the copies of this gene, were all subacrocentric and were among the largest, measuring about 4-4.5 μm long. The sites were observable in both the condensing and condensed mitotic chromosomes as well as in the interphase nuclei (Fig. 3a-h). Signals on two of the sites on one pair of homologous chromosomes (Fig. 3a and g) were quite strong while the other two sites on another pair of homologous chromosomes were slightly less vivid. There was no difference in the number of this large ribosomal gene between the two cultivars.

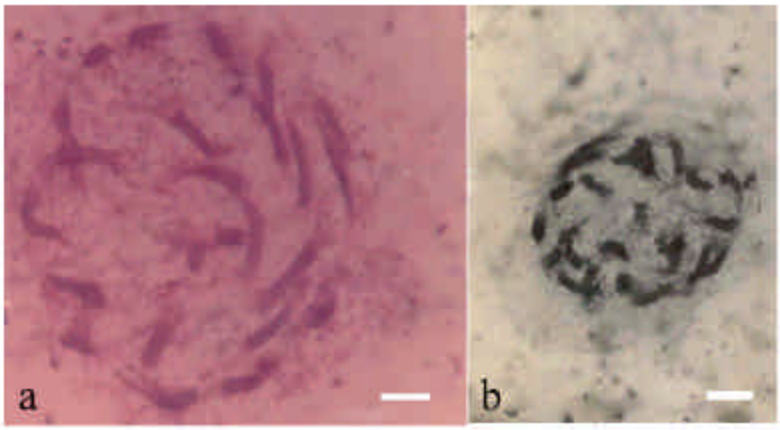

Fig. 2: Mitotic mid and late prophase chromosomes of *C. mannii* showing small chromosomes and non-uniform chromatin condensation; a) mid prophase and b) late prophase nuclei. Scale bar represents 5 μm

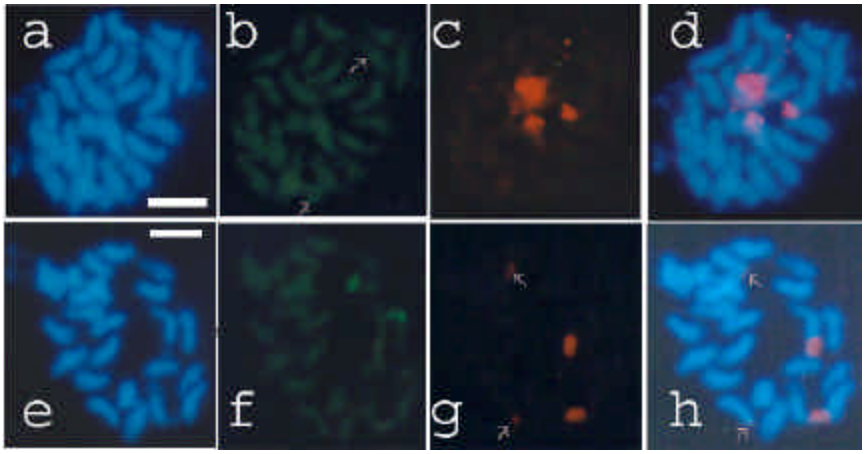

Fig. 3: *In situ* hybridization sites of 5S and 18S-5.8S-25S rDNA sequences on mitotic chromosomes of the two cultivars of *C. mannii*; a) DAPI image of mitotic chromosomes of the cultivar with oblong-round fruit; b) mitotic chromosomes showing two sites of 5S rDNA (arrows) and c) four sites of 18S-5.8S-25S rDNA; d) transparent overlay of c on a; e) DAPI stained chromosomes of the cultivar with cylindrical fruit; f) two sites of the 5S rDNA and g) four sites of the 18S-5.8S-25S rDNA comprising two major and two minor (arrows) sites; h) transparent overlay of gone. Scale bar represents 5 μm

The 5S rRNA gene: Two homologous chromosomes had sites of the 5S rRNA gene (Fig. 3a-h). The chromosomes were mostly submetacentric and measured about 4 μm long. The sites were subterminal on the two chromosomes. The strength of the signals was considerably obvious though within the lower limits of sensitivity of detection. There was no double site in a location and no two sites on the same chromosome. No site of this gene was noticed on a chromosome carrying the larger 18S-2.5S-25S rRNA gene.

DISCUSSION

One major problem with cucurbits, especially *C. mannii* is the tendency of dividing cells to relapse to interphase if disturbed during harvest of root tips for chromosomal investigations. This is apparently the cause of the regular low yield of metaphase cells in this species and other cucurbits. The problem of small chromosome size and non amenability to cytological treatments (Whitaker, 1974; Jeffrey, 1980; Okoli, 1984) in the cucurbit

family has constituted a serious obstacle to karyological and genomic understanding and characterization of members of the family. The relatively small size of the chromosomes compared with those of the Brassicaceae (Maluszynska and Heslop-Harrison, 1993), Araceae (personal observation), Poaceae (Leitch and Heslop-Harrison, 1993) constitutes a major handicap to its cytology. These problems may have contributed to the incorrect count of 2n = 22 reported by Jeffrey (1980). The authentic chromosome count of 2n = 24 established in this work for both cultivars is therefore the correct somatic (root tip) chromosome number for *C. mannii*. The chromosomes were about similar size range with chromosomes of *Musa* spp. though slightly larger (Osuji *et al*., 1998), the only variation being that the difference in length between the largest and smallest chromosomes is less in *C. mannii*.

The occurrence of rDNA in the genome of *C. mannii* shows that these sequences are ubiquitous among the angiosperms. The 18S-5.8S-25S rDNA is often associated with chromosomes which have secondary constrictions and attendant nucleolar organizers (Osuji *et al*., 1998). Hence occurrence of four sites of 18S-5.8S-25S rDNA indicates the presence of four chromosomes with secondary constrictions and nucleolar organizers. The large signal sizes at the sites of this gene indicate a large number of tandem insertions (i.e., multiple copies) of the individual gene at the fluorescent sites. The variation in signal strength of the 5S rRNA gene is an indication that the number of copies per site and their degree of expression may differ in the same genome.

The number of sites of the 18S-5.8S-25S rDNA does not have direct obvious relationship with ploidy or with the genome size. For instance, rye, with 2n = 14 has only a single pair of sites while the related barley, which also has 2n = 14, has six pairs of sites comprising two major and two minor sites (Leitch and Heslop-Harrison, 1993). Diploid *Brassica* species have variable number of 18S-5.8S-25S rDNA sites (Maluszynska and Heslop-Harrison, 1993). In the Fabaceae, *Vigna unguiculata*, 2n = 2*x* = 22, had four major and one minor (Galasso *et al*., 1995) while *Vicia faba*, 2n = 2*x* = 12, has only one major pair (Pearce *et al*., 1996) of sites of 18S-5.8S-25S rDNA. But in *Musa*, the number of sites of the 18S-5.8S-25S rDNA reflects ploidy while the number of sites of the 5 S rDNA varies within cultivars at similar ploidy level (Osuji *et al*., 1998). In *Arabidopsis thaliana*, sequences coding for 5S rDNA have been mapped to the short arm of chromosome 4 (Murata, Heslop-Harrison and Motoyoshi, 1997) while sequences coding for 18S-5.8S-25S were not detected on this chromosome (Thompson *et al*., 1996a,b).

In the tropics and subtropics, curcurbits constitute a major source of fruits and vegetables for healthy dieting. Whereas some species have evolved as major crops, some others are rather diminishing in importance and relevance owing to poverty and other economic factors. *Cucumeropsis mannii* is one of the cucurbits that have been selected against due to their inability to compete favourably with rival species and cultivars. For instance, *Citrullus lanatus* (commonly called egusi) is an alternative crop as an item for soup thickening but obviously yields more fruits and seeds than *C. mannii*. *Cucurbita moschata* (pumpkin) is eaten as porridge with yam in just the same way *C. manni* is eaten with yam. But *C. moschata* yields edible fruits, seeds and leaves, which altogether make it more favoured. For these reasons, a combination of *C. lanatus* and *C. moschata* out compete *C. Mannii*. Hence the very existence of *C. mannii* is threatened despite its food and medicinal values. This crop species might get extinct in the next ten years if nothing is done to conserve, characterize and exploit the benefits of its gene pool. The species could serve as a source of novel gene(s) for future plant improvement programme(s).

REFERENCES

Andres, T.C., 2004. Website for the plant family Cucurbitaceae and home of the Cucurbit Network. http://www.cucurbit.org/family.html.

Galasso, I., T. Schmidt, D. Pignone and J.S. Heslop-Harrison, 1995. The Molecular Cytogenetics of *Vigna unguiculata* (L) Walp: The Physical Organization and Characterization of 18S-5.8S-25S rRNA Genes, 5s rRNA Genes, Telomere-like Sequences and a Family of Centromeric Repetitive DNA Sequences. Theor. Applied Gene., 91: 928-935.

Gbile, Z.O., 1986. Ethnobotany, Taxonomy and Conservation of Medicinal Plants. In: Sofowora, A. (Eds.). the State of Medicinal Plants Research in Nigeria. University of Ibadan Press, Nigeria, pp: 13-29.

Gerlach, W.L. and J.R. Bedbrook, 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res., 7: 1869-1885.

Gerlach, W.L. and T.A. Dyer, 1980. Sequence organization of the repeating units in the nucleus of wheat which contains 5S rRNA genes. Nucleic Acids Res., 8: 4851-4865.

Gill, L.S., 1992. Ethnomedical Uses of Plants in Nigeria. Uniben Press, University of Benin, Nigeria, pp: 276.

Jeffrey, C., 1980. A review of Cucurbitaceae. Bot. J. Linn. Soc., 1: 233-247.

King, R.C., 1974. Handbook of Genetics. Plenum Press, New York.

Leitch, I.J. and J.S. Heslop-Harrison, 1993. Physical mapping of four sites of 5S rDNA sequences and one site of alpha-amylase-2 gene in barley (*Hordeum vulgare*). Genome, 36: 517-523.

Maluszynska, J. and J.S. Heslop-Harrison, 1993. Physical mapping of rDNA in *Brassica* species. Genome, 36: 774-781.

Murata, M., J.S. Heslop-Harrison and F. Motoyoshi, 1997. Physical mapping of the 5S ribosomal RNA genes in *Arabidopsis thaliana* by multicolour fluorescence *in situ* hybridization with cosmid clones. Plant J., (In Press).

Okigbo, B.N., 1975. Neglected plants of horticultural and nutritional importance in traditional farming systems of tropical Africa. Acta Hortic., 53: 131-150.

Okoli, B.E., 1984. Wild and cultivated cucurbits in Nigeria. Econom. Bot., 38: 350-357.

Osuji, J.O., J. Crouch, G. Harrison and J.S. Heslop-Harrison, 1997. Identification of the genomic constitution of *Musa* L. lines (bananas, plantains and hybrids) using molecular cytogenetics. Ann. Bot., 80: 787-793.

Osuji, J.O., J. Crouch, G. Harrison and J.S. Heslop-Harrison, 1998. Molecular cytogenetics of *Musa* species, cultivars and hybrids: Location of 18S-5.8S-25S and 5S rDNA and telomere-like sequences. Ann. Bot., 82: 243-248.

Osuji, J.O., 2003. Cytogenetic techniques. In: Onyeike, E.N. and J.O. Osuji (Eds.). Research Techniques in Biological and Chemical Sciences. Springfield Publishers Ltd., Owerri, Nigeria, pp: 70-83.

Pearce, S.R., G. Harrison, M. Wilkinson, D. Li, J.S. Hesslop-Harrison, A.J. Flavell and A. Kumar, 1996. The Ty1-copia group retrotransposons in *Vicia* species: Copy number, sequence heterogeneity and chromosomal localization. Mol., Gen. Genet., 250: 305-315.

Ramchandran, C., U.S. Seshadri and R.A. Pai, 1983. Giemsa C-banding in Cucumis. In: Abstracts of contributed papers, Part II, XV Intl. Congr. Genetics. IBH Publishers, New Delhi, Oxford, pp: 685.

Roy, R.P., S. Saran and B. Dutt, 1991. Cytogenetics of Cucurbitaceae. In: Tsuchiya, T. and P.K. Gupta (Eds.). Chromosome Engineering in Plants: Genetics, Breeding and Evolution. Elsevier, New York, pp: 181-199.

Schwarzacher, T., A.R. Leitch, M.D. Bennett and J.S. Heslop-Harrison, 1989. *In situ* localization of parental genomes in wide hybrid. Ann. Bot., 64: 315-324.

Sofowora, A., 1986. The State of Medicinal Plants Research in Nigeria. Ibadan University Press, Nigeria, pp: 20.

Thompson, H., R. Schmidt, A. Brandes, J.S. Heslop-Harrison and C. Dean, 1996a. A novel repetitive sequence associated with the centromeeric regions of *Arabidopsis thaliana*. Mol. Gen. Genet., 253: 247-252.

Thompson, H., R. Schmidt and C. Dean, 1996b. Identification and distribution of seven classes of middle-repetitive DNA in the *Arabidopsis thaliana* genome. Nucleic Acids Res., 24: 3017-3022.

Whitaker, T.W., 1974. *Cucurbita.* In: King, R.C. (Eds.). Handbook of Genetics. New York, pp: 135-143.

Willhoeft, U., 1997. Fluorescent *in situ* hybridization of ribosomal DNA to mitotic chromosomes of tsetse flies (Diptera: Glossinidae: *Glossina*). Chromosome Res., 5: 262-267.

Zoro Bi, I.A., K.K. Koffi and Y. Djé, 2003. Botanical and agronomic characterization of three species of cucurbit consumed as sauce in West Africa: *Citrullus* sp., *Cucumeropsis mannii* Naudin and *Lagenaria siceraria* (Molina) Standle. Biotechnol. Agron. Soc. Environ., 7: 189-199.