Short Scientific Report

doi: 10.25081/jpc.2019.v47.i3.6056
Journal of Plantation Crops, 2019, 47(3): 197-201

Chromosome number analysis in different sex types and open-pollinated seedlings of nutmeg (*Myristica fragrans* Houtt)

R. Ramakrishnan Nair*

ICAR-Indian Institute of Spices Research, Marikunnu PO, Kozhikode-673012, Kerala, India

(Manuscript Received: 26-04-2019, Revised: 17-10-2019, Accepted: 25-10-2019)

Keywords: Chromosome number, female tree, holocentric chromosomes, male tree, monoecious tree

Myristica fragrans Houtt. (Myristicaceae), commonly known as nutmeg tree, is a dioecious evergreen tree, yields the nutmeg seed of commerce and mace the aril covering the seed, both are widely used as spices. A native of Moluccas Islands (Indonesia), nutmeg has a pleasant fragrance and warm taste which makes this spice unique. It is one of the popular flavour ingredients in processed food, vegetables, beverages and also known for medicinal uses (Olaleye *et al.*, 2006; Haldankar *et al.,* 2008). Even though dioecious usually, monoecious trees having variable sex expression are occasionally found in nutmeg (Flach, 1966; Haldankar *et al.*, 2008). Three chromosome numbers have been reported for nutmeg such as 2n=38 (Dhamayanthi and Krishnamoorthy, 1999), 2n=42 (Simmonds, 1954; Purseglove *et al*., 1981) and 2n=44 (Flach, 1966). The present study aims to resolve the contradictions in chromosome numbers reported in nutmeg by counting mitotic metaphase plates from different sex types and open-pollinated seedlings.

One tree each of female, male and monoecious sex having more male flowers and occasional female flowers maintained at ICAR-Indian Institute of Spices Research, Calicut, India, were used as a source of root samples for chromosome analysis. For analysis of seedlings, fully matured and ripened fruits from open pollination were collected from the female plant and seeds were removed. Fresh seeds were sown in plastic pans filled with fresh and clean river sand and watered as per requirement. After germination (40 d after sowing) and on attaining two-leaf stage, six plants were randomly selected and root tip samples of the same were analyzed to assess chromosome number.

Roots tips having active growth and of 5-10 mm length were collected from the adventitious roots of ten years old trees of different sexes, between 11.00 and 11.30 AM. For sample collection from seedlings, the seed pan was watered sufficiently to make the river sand loose. Six plants were carefully pulled out at random, without damaging the root system and actively growing root tips were collected as described above.

Samples were pretreated with a mixture of saturated paradichlorobenzene solution and 2 mM 8-hydroxyquinoline in 1:1 ratio for 4h at 4-5 °C. Pre-treated samples were washed thoroughly in double-distilled water and hydrolyzed with 5 N HCl at 0 °C for 4 min. Hydrolyzed root tips were rinsed in double distilled water and subsequently stained in 2 per cent acetoorcein for 16 h. Temporary squash preparations were made in 45 per cent acetic acid. A separate set of the root tips were fixed in 3:1ethyl alcohol and acetic acid for 24 h, after pre-treatment and squash preparation was made as described above. As the former technique yielded comparatively good chromosome preparations, the same was followed throughout the study.

**Corresponding Authoro: rrnair9@rediffmail.com*

Observations were performed under 100x objective of a DMRB (Leica, Germany) research microscope and photomicrographs were taken using a Moticam-2300 (Motic, China) digital microscope camera. The image was captured and saved using Motic Images plus-2.0 photo capturing software, after the calibration of magnification. Metaphase plates of about 6 to 31 with good spread of chromosomes from 3-6 slides were counted for chromosome number of different plant types analyzed. The percentage of cells showing a particular chromosome number was calculated for each sex type and seedlings analyzed.

Of the two squash techniques tested, the one involved direct hydrolysis and staining after pre-treatment resulted in more clear mitotic plates. Among the materials stained, the root tip cells from seedlings showed better staining of the chromosomes compared to those from mature trees. Unlike the earlier reports (Flach, 1966; Dhamayanthi and Krishnamoorthy, 1999) an easy squash technique is standardized for nutmeg, avoiding the fixation of root tips subsequent to pre-treatment and proceeding directly for hydrolysis and staining. It appeared that the fixation negatively affected the maceration of nutmeg root tips and root tips became brittle, which resulted in preparations with poor chromosome spread and staining. It is possible that the compounds present in the nutmeg root tips interact with the fixative and producing substances interfering with the maceration and staining. The better staining of the root tips from seedlings may be mainly due to the easy penetration of stain in the relatively soft tissues of the thin roots from seedlings compared to the thick root tips excised from mature trees which has more differentiated tissues.

Of the total number of mitotic metaphase plates counted, 58.06 per cent in female plants, 72 per cent in male plants and 83.33 per cent monoecious plants showed 2n=44 as the chromosome number. A typical mitotic metaphase plate showing 2n = 44 is presented in Fig.1a. Variant cells with 2n=41, 2n=42, and 2n=45 observed in low frequencies (4-25.81%) among these different sex types. Among the six seedlings analyzed 42.86 to 100 per cent plates in different seedlings showed 2n=44 as the chromosome number. Variant numbers observed among seedlings were 2n=43, 2n=45, and 2n=46. One seedling (Seedling 2) showed a number of variant cells (57.14%) compared to others. The distribution of chromosome numbers among the different sex types and seedlings analyzed is presented in Table 1. Mitotic metaphase plates with variation in chromosome number are presented in Figure 1a-e. The late metaphase and early anaphase plates of the nutmeg showed ring-like configuration of replicated chromosomes, unlike the chi-like appearance in conventional mitosis (Fig. 2a-b). Delayed segregation of chromosomes was also observed among the late mitotic stages (Fig. 2c).

Table 1. Somatic chromosome number in different sex types and open pollinated seedlings of *Myristica fragrans* Houtt.

Plant Identity	Number of cells observed	Frequency of chromosome numbers observed					
		2n=41	2n=42	2n=43	2n=44	2n=45	2n=46
Female	31	8 (25.81)	5 (16.13)	-	18(58.06)	-	-
Male	25	2 (8.00)	4 (16.00)	-	18 (72.00)	1(4)	-
Monoecious	18	3 (16.67)	-	-	15 (83.33)	-	-
Seedling 1	9	-	-	2 (22.22)	5 (55.56)	-	2 (22.22)
Seedling 2	7	-	-	1(14.285)	3 (42.86)	2 (28.57)	1(14.285)
Seedling 3	6	-	-	-	6 (100)	-	-
Seedling 4	7	-	-	-	7(100)	-	-
Seedling 5	10	-	-	-	8(80)	2(20)	-
Seedling 6	8	-	-	-	7(87.5)	1(12.5)	-
Total	121	13 (10.74)	9 (7.44)	3(2.48)	87(71.90)	6 (4.96)	3 (2.48)

*Values in parentheses indicate percentage

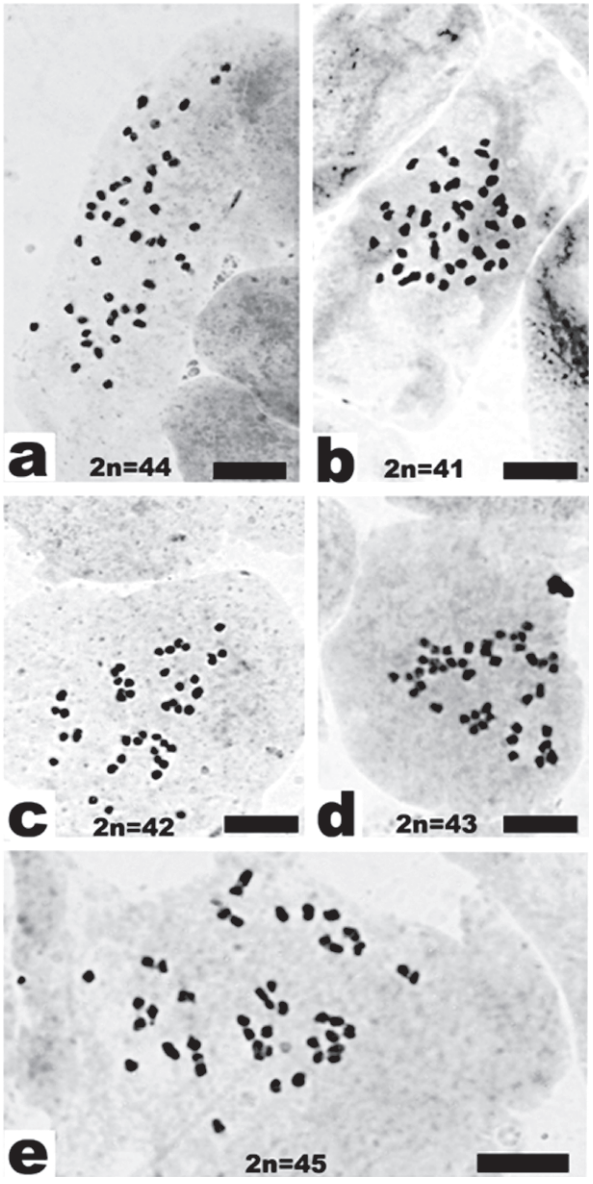

Fig. 1. Chromosome number in nutmeg

a. A typical mitotic metaphase cell from root tip of female tree showing 2n=44

b. A variant cell from monoecious tree showing 2n=41

c. A variant cell from male tree showing 2n=42

d. A variant cell from Seedling-1 showing 2n=43

e. A variant cell from Seedling-2 showing 2n=45

Bars represent 5 µm in a-e.

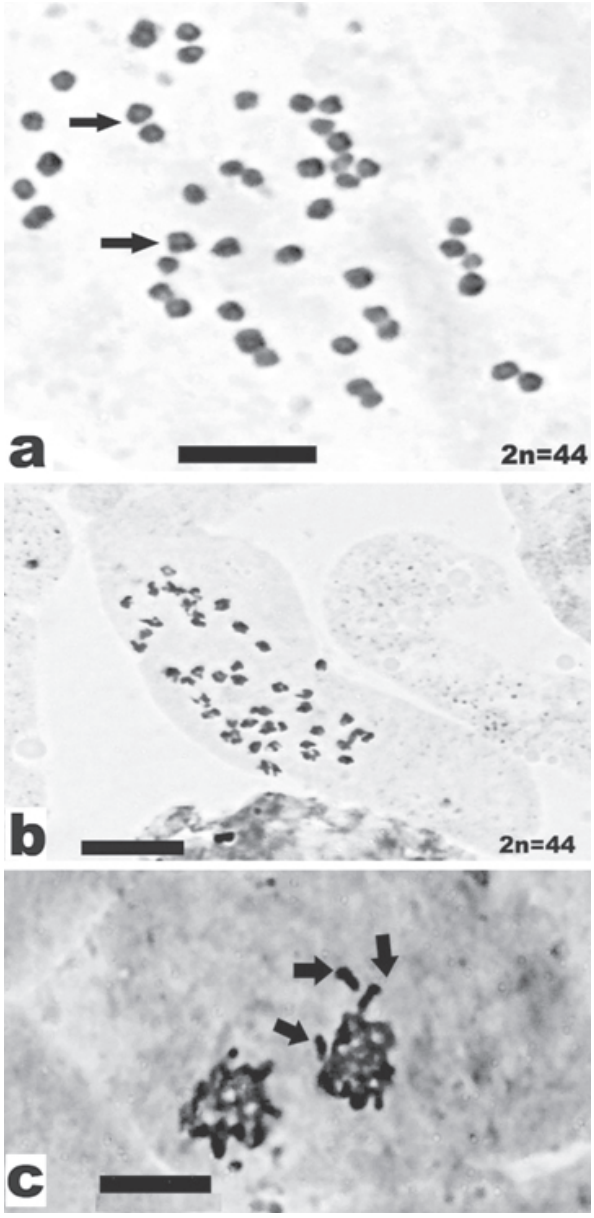

Fig. 2. Late metaphase and early anaphase showing ring – like orientation of divided mitotic chromosomes and telophase showing lagging of chromosomes

a. Late metaphase showing ring like chromosomes (→)

b. Early anaphase showing segregation of chromosomes in the ring

c. Telophase showing migration of lagged chromosomes toone pole (→)

Bars represent 5 µm in a, b and c

The chromosome number in nutmeg has been reported differently by the earlier workers (Simmonds, 1954; Flach, 1966; Purseglove *et al*., 1981; Dhamayanthi and Krishnamoorthy, 1999). Simmonds (1954) reported the chromosome number of *M. fragrans* as 2n= 42. Purseglove et al. (1981) stated the basic chromosome number of the genus as 7 and chromosome number of *M. fragrans* as 2n= 42. Subsequent investigations by Flach (1966) revealed that the most frequently occurring chromosome number in nutmeg is *2n*= 44 in mature trees of known sex as well as seedlings and some cells showed deviations such as 2n= 45 and 46. He opined that the squash technique to determine chromosome number is not suitable for nutmeg and determined chromosome number through microtome sectioning. He suggested the holocentric nature of chromosomes in nutmeg. Dhamayanthi and Krishnamoorthy (1999) reported somatic chromosome number in nutmeg seedlings as 2n= 38 by a squash technique developed by them using 0.5 per cent colchicine as pre-treating agent and lactopropionic orcein as stain.

The chromosome counts of the present study support the findings of Flach (1966) that chromosome number in different sex types and seedlings of nutmeg is 2n=44. He also observed variant numbers like 2n=45 and 46 occasionally. He attributed this to the splitting of chromosomes. However, in the present study cells with variant numbers were observed in all plant types analyzed. The other chromosome number reports of 2n=42 (Simmonds, 1954) and 2n=38 (Dhamayanthi and Krishnamoorthy, 1999) for nutmeg might have originated based on counting very limited number of mitotic metaphase plates. The nutmeg tree is among the plant species having holokinetic (holocentric) chromosomes due to the presence of diffused centromere (Flach, 1966). This might be helpful for perpetuating chromosome fragments as individual chromosomes. Delayed segregation of chromosomes was also observed among the late mitotic stages in the present study. This may also be attributed to generating cells with aneuploid number of chromosomes.

Holocentric chromosomes have been reported in species of monocot plant families like *Cyperacea*e, *Juncaceae*, *Chionographis,* and dicots like *Cuscuta* subgenus Cuscuta, *Drosera* (Heckmann and Houben, 2013) as in *Myristica fragrans* (Flach, 1966). The probability of holocentric chromosome fragments being transmitted during nuclear divisions is high compared to their monocentric counterparts. Stable transmission of artificial chromosome rearrangements in different holocentric plants during mitosis and meiosis proved that the fragments of chromosomes retain the centromeric activity (LaCour, 1953; Hakansson, 1954; Nordenskiold, 1963). Chromosome number variation in species with holocentric chromosomes has been reviewed by Luceno and Guerra (1996).

Aneusomatic chromosome number variation has also been recorded in plant species having monocentric chromosomes (Hegwood and Hough, 1958; Mix *et al*., 1978, Nair and Ravindran, 1994; D' Amato, 1997; Nair, 2007). Hegwood and Hough (1958) observed chromosome number mosaicism in somatic tissues of White Winter Pearmain apple and 6 of its seedlings. A high frequency of basic euploid chromosome numbers and cells with both higher and lower numbers than the euploid mode at random were observed in dividing cell layers of shoot buds. In root tips of microspore derived plants of barley, Mix *et al*. (1978) observed cells of different ploidy levels. Aneusomatic variation in root tip cells of *Vanilla planifolia* was reported by Nair and Ravindran (1994). In *Piper magnificum*, besides the normal diploid chromosome number of 2n=26 in higher frequency, cells with 2n= 24, 25, 27 and 28 were also observed in lower frequency in cells of the same root tip (Nair, 2007). D' Amato (1997) indicated that aneusomatic variation in chromosome number is present in natural populations of *Orobanche gracilis*, *Poa pratensi s*and *Claytonia virginica.* In all the above-cited examples the reason for aneusomatic variation has been attributed to abnormalities during mitosis. However, heritable nature and thereby genetic control of such variation have been indicated in few cases (Hegwood and Hough, 1958; Ogura, 1978). As cells with aneusomatic variation in number of chromosomes were observed in all the categories of plants analyzed in the present study, genetic control of the phenomenon can be reasonably suspected.

The ring-like appearance of replicated chromosomes at late metaphase indicates that in nutmeg even though the chromosomes have diffused centromeres, telomeres have more centromere-like activity, which resulted in these ring-like configurations of chromosomes. Clustered distribution of heterochromatin in

holocentric chromosomes has been indicated in *Drosera* (Sheikh and Kondo, 1995), *Luzula elegans* (Ray and Venketeswaran, 1979), *Rhynchospora* (Vanzela and Guerra, 2000), and *Cuscuta approximate* (Guerra and Gracia, 2004) based on analysis of Giemsa banding. Preferential distribution of heterochromatic blocks on terminal and sub-terminal regions are most common, although some central blocks are found. It is possible that such concentration of heterochromatic blocks is existing towards the telomeric region of *M. fragrans* chromosomes also, the result of which the telomeric regions are late replicating during chromosome replication and attain a ring-like configuration during late metaphase while polar segregation of chromatids initiated.

In view of the present observations, the chromosome number in nutmeg may be accepted as 2n=44 as reported earlier by Flach (1966). As an easy squash technique is standardized, identification of the sex of the seedlings at juvenile stage is possible on availability of a sex-specific in situ-hybridization kit.

Acknowledgements

Research facilities provided by the Director, ICAR-Indian Institute of Spices Research and plant materials provided by Mr. B. Krishnamoorthy, former Head of Crop Improvement Division are thankfully acknowledged.

References

D' Amato, F. 1997. Role of somatic mutations in the evolution of higher plants. *Caryologia* **50**(1): 1-15.

Dhamayanthi, K.P.M. and Krishnamoorthy, B. 1999. Somatic chromosome number in nutmeg (*Myristica fragrans* Houtt.). *Journal of Spices and Aromatic Crops* **8**(2): 205-206.

Flach, M. 1966. Nutmeg cultivation and its sex problem: An agronomical and cytogenetical study of the dioecy in *Myristicafragrans* Houtt. And *Myristica argentena* Warb. [Ph.D Thesis][Wageningen]: Mededelingen Landbouwhoge school Wageningen (66-1), Dissertation No Flach 397, Veenman: Wageningen.

Guerra, M. and Gracia, M. A. 2004. Heterochromatin and rDNA sites distribution in the holocentric chromosomes of *Cuscuta approximate* Bab. (Convolvulaceae). *Genome* **47**(1):134-140.

Haldankar, P. M., Khandekar,R. G., Rangwala, A. D. and Joshi, G.D. 2008. Nutmeg. In: *Spices Vol. 2.* (Eds). Parthasarathy, V. A., Parthasarathy, U. and Kumar, A. Today and Tomorrow Publishers, New Delhi, pp. 203-246.

Hakansson, A. 1954. Meiosis and pollen mitosis in x-rayed and untreated spikelets of *Eleocharis palustris*. *Hereditas* **40**(3-4) : 325-345.

Heckmann, S. and Houben, A. 2013. Holokinetic centromeres In: *Plant Centromere Biology.* (Eds). Jiang, J. and Brichler J. A. John Wiley and Sons Inc, Iowa pp. 83-94.

Hegwood, M. P. and Hough, L. F. 1958. A mosaic pattern of chromosome numbers in the white winter pearmain apple and six of its seedlings. *American Journal of Botany* **45**(5): 349-354.

LaCour, F. L. 1953. The Luzula system analysed by x-rays. *Heredity* **6**(Suppl.):77-81.

Luceno, M. and Guerra, M. 1996. Numerical variations in species exhibiting holocentric chromosomes: A nomenclatural proposal. *Caryologia* **49** (3-4):301-309.

Mix, G., Wilson, H. M. and Foroughi-Wehr, B. 1978. The cytological status of plants of *Hordeum vulgare* L. regenerated from microspore callus. *Z. Pflanzenzüchtg*. **80**(1): 89-99.

Nair, R. R. and Ravindran, P. N. 1994. Somatic association of chromosomes and other mitotic abnormalities in *Vanilla planifolia* (Andrews). *Caryologia* **47**(1): 65-73.

Nair, R. R. 2007. Aneuploid variation of chromosome number in the somatic cells of *Piper magnificum* Trel. *Cytologia* **72**(2): 239-242.

Nordenskiold, H. 1963. A study of meiosis in progeny of x-irradiated *Luzula purpurea*. *Hereditas* **49**(1-2):33-47.

Ogura, H. 1978. Genetic control of chromosomal chimerism found in a regenerate from tobacco callus. *Japanese Journal of Genetics* **53**(2): 77-90.

Olaleye, M.T., Akinmoladun, A.C. and Akindahuns, A.A. 2006. Antioxidant properties of *Myristica fragrans* (Houtt) and its effect on selected organs of albino rats. *African Journal of Biotechnology* **5**(13):1274-1278.

Purseglove, J. W., Brown, E. G., Green,C. L. and Robbins, S. R. J. 1981. Nutmeg and Mace. Spices Vol. 2. Longman, London, pp. 174-228.

Ray, J. M.and Venketeswaran, S. 1979. DNA replication, 3H-cRNA in situ hybridizationand c-band patterns in the polycentric chromosomes of *Luzula purpurea* Link. *Chromosoma* **74 (**3): 337-346.

Sheikh, S. A. and Kondo, K. 1995. Differential staining with Orcein, Giemsa, Cma and Dapifor comparative chromosome study of 12 species of Australian *Drosera* (Droseraceae). *American Journal of Botany* **82**(10): 1278-1286.

Simmonds, N. W. 1954. Chromosome behaviour in some tropical plants. *Heredity* **8**(1):139-146.

Vanzela, A. L. L. and Guerra,M.2000. Heterochromatin differentiation in holocentric chromosomes of *Rhyncospora* (Cyperaceae). *Genetics and Molecular Biology* **23**(2):453-456.