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Assessment of genetic variation among 14 ecotypes of *Nyctanthes arbortristis* L. collected from western Odisha using cytological and DNA markers

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ABSTRACT

The genetic divergence among 14 ecotypes of *Nyctanthes arbortristis* (L.) were assessed using karyotype, meiotic studies and RAPD analysis. Mitotic and meiotic analysis confirmed 2n = 44 chromosomes. Somatic chromosome analysis revealed symmetric karyotype with prevalence of median and sub-median chromosomes with TF% ranging from 39.8 to 44.08. Meiotic analysis of 14 ecotypes also showed 22 bivalents in majority of PMCs, but in some PMCs bivalents (19.4±1.03 to 21.2±0.24), univalents (2.1±1.06 to 3.9±1.48) and quadrivalents (0.36±0.14 to 1.6±0.24) were also appeared in combination. The PMCs revealed high proportion of ring bivalents over rod bivalents with terminalised chiasmata, and terminalization coefficient ranged from 0.895 to 0.919. Although PMCs showed normal 22:22 segregation of chromosomes to two poles during Anaphase-I, univalents in the form of laggards were often observed. Pollen fertility and percentage of seed germination were low in all ecotypes which was indicative of partial genetic heterozygosity. RAPD marker profile generated by 10 RAPD primers showed moderate polymorphism among the ecotypes with Jaccards similarity indices ranging from 0.648 to 0.962 among the ecotypes of *N. arbortristis*. The clustering of ecotypes based on RAPD data also agreed with chromosome behaviour, pollen viability and germination of seeds. Moreover, these findings have many implications for future *in vitro* studies aimed at development of genotypes with higher content of metabolites (rengyolone, urosolic acid, arbortristosides and nyctanthic acid) of pharmaceutical importance.

1. Introduction

Nyctanthes arbortristis (L.), belongs to the family-Oleaceae and is native to India. It is an important medicinal plant commonly known as Parijatha (Sanskrit), Night Jasmine (English) and Gangasiuli (Odia). This plant is a large shrub or small tree, with flaky grey bark, stiff whitish hair on young branches and rough leaves (Agarwal and Pal, 2013). The flowers are fragrant having six white petals with an orange centre (Ratnasooriya *et al.*, 2005). It is also called as *Harsinghar* (sad tree) because its flowers open at dusk and fall down at dawn from the tree. Different plant parts of *N. arbortristis* are used as medicine for treatment of various ailments because it possesses anti-malarial (Kumari *et al.*, 2012; Nagendrappa *et al.*, 2013; Agarwal *et al.*, 2013; Godse *et al.*, 2016), antibacterial (Khatune *et al.*, 2001), anti-helminthic, anti-inflammatory (Saxena *et al*., 1984; Das *et al.*, 2008; Nirmal *et al.*, 2012), hepato-protective (Hukkeri *et al.*, 2006), immune-potential, antipyretic (Saxena *et al.*, 1987), antifungal (Gyanchandani *et al.*, 2000), immunomodulatory (Khan *et al.*, 1995; Bharshiv *et al.*, 2016), anti-leishmanial (Tandon *et al.*, 1991; Shukla *et al.*, 2011; Shukla *et al.*, 2012) properties Biologically important metabolites such as alkaloids, phytosterols, phenolics, tannins, flavonoids, glycosides and saponins were isolated and characterized from this plant (Priya and Ganjewala, 2007; Rahman *et al.,* 2011).

Genetic diversity assessment among natural populations of medicinal species is also quite important in the perspective of their phenotypic plasticity (Geng *et al.*, 2016) vis-a-vis their evolution, domestication and conservation. The ecotypes as well as the populations of *N. arbortristis* are quite heterogeneous in their respective natural habitat, and they are found to be distributed across the tropical and semiarid tropical regions either as ornamental plant or as

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wild tree. Therefore, estimation of genetic variation among the genotypes of this medicinal species is crucial prior to its conservation, domestication and implementation of genetic improvement strategies. Keeping this as requirement, various tools including morphological, cytological, biochemical and molecular markers have been used for genetic diversity studies in many medicinal species including *Osmanthus fragrans* (Duan *et al.*, 2013; Hu *et al.*, 2014), *Forsythia* species (Chung *et al.*, 2013), *Fraxinus* species (Sollars *et al.*, 2017) . In *N. arbortristis*, the cytological exploration mostly limited to somatic chromosome count and chromosome behavior pertaining to cytotaxonomical status. The somatic chromosome number of *N. arbortristis* has been reported from time to time as 2n = 44 (Bolkhorskikh *et al.*, 1969; George and Geethamma, 1984; George *et al.*, 1989) and as 2n = 46 (Kundu and De, 1968). During last couple of decades, DNA markers have been deployed as supplement for conventional biometric tools for genetic studies in many species (Sahu *et al.*, 2016). Recently, Rohilla *et al.* (2017) used 40 RAPD primers and assessed the genetic diversity among 16 ecotypes of *N. arbortristis* obtained from Northern-Central India.

Therefore, in this study an attempt has been made to assess the genetic diversity among the ecotypes of *N. arbortristis* collected from different parts of western Odisha using karyotype analysis, meiotic chromosome behavior and RAPD marker analysis.

2. Materials and methods

2.1 Plant materials

Fourteen ecotypes of *N. arbortristis* were collected from different parts of western Odisha (Table 1) and planted in the botanical garden of School of Life Sciences, Sambalpur University, Odisha, India. The roots of sprouted seedlings, flower buds and leaf samples were used for mitotic, meiotic and DNA marker analysis, respectively.

2.2 Mitotic analysis and 4C DNA estimation

Well developed roots (1-3 cm) obtained from the seedlings at 7.00-8.00 a.m. and pre-treated with saturated pre-chilled p-dichlorobenzene (PDB) solution for two hours at 20ºC, followed by fixation in 1:3 aceto-alcohol and kept overnight at room temperature. Subsequently, the root tips were transferred to 70% ethanol and stored at 4^0C. Hydrolysis of the root tips were carried out in preheated 1N HCl at 60^0C for 10 min followed by staining with the help of 1.5% aceto-orcein for one hour and squashed with 45% propionic acid. Suitable metaphase plates were observed under compound microscope (Unilab, India) and were documented using Nikon Coolpix-4500 camera. The images of metaphasic plates were analyzed manually as suggested by Fukui (1986) and idiogram was prepared. The chromosomes were classified as median (m), submedian (sm), subterminal (st) and terminal (t) according to the position of the centromere (Hirahar and Tatuno, 1967) and on the basis of chromosome length (long: e"3.0 µm, medium: e" 2.0 to < 3.0µm and Small: < 2.0µm). The chromosomes were morphologically grouped into following types- type A: Long median (A_M), type B: Medium median (B_M), type C: Medium submedian (C_{SM}), type D: Medium subterminal (D_{ST}), type E: Short median (E_M) and type F: Short submedian (F_{SM}). Total chromatin length, fraction of short arms in total chromatin length (TF%) and relative length of shortest chromosome compared to longest (S%) were also calculated.

Actively growing root tips of fourteen ecotypes of *N. arbortristis* and that of *Allium cepa* (as control) were excised and fixed and stored as explained above. These root tips were thoroughly washed and hydrolyzed for 10 min with 3N HCl at room temperature. After another wash the hydrolyzed tips were transferred to Feulgen stain (pH 3.2) for two hours at room temperature and squashed under a coverslip in glycerol. Four slides from each sample were made and readings in arbitrary units of 40 cells were obtained for each ecotype and control sample at 550 nm using 20/30 PV microspectrophotometer (Craic Technologies, USA). Only 4C nuclei at mid-prophase were measured and the arbitrary units were converted to picogram (pg) of DNA per nucleus by taking the mean of identical number readings of *A. cepa* root tips and its 4C nuclear DNA content (67.1 pg) as standard (Bennet *et al.*, 2000).

2.2 Meiotic analysis

The flower buds of appropriate size were fixed in 1:3 aceto-alcohols and kept for 24 h at 25 ± 2ºC and then it was transferred to 70% ethanol for and stored at 4ºC. The anthers of suitable size were squashed in a drop of 1.5% acetocarmine, and the meiotic behaviour of chromosomes, including chromosome association and segregation, were observed. An average of 25-30 Pollen Mother Cells (PMCs) of each ecotype were analyzed at diplotene/diakinesis/ metaphase-I stages. During anaphase-I, about 20 cells was analyzed and the segregation pattern of chromosomes was observed. The pollen viability was assessed by staining the pollen grains with 1:1 acetocarmine: glycerine.

2.3 RAPD marker analysis

Young apical leaves were obtained from 14 ecotypes and ground under liquid nitrogen to fine powder, and genomic DNA was isolated following the modified CTAB method and purified using RNAse and proteinase K treatment followed by 24 chloroform: 1 isoamyl alcohol washes

(Mishra *et al.*, 2013). The DNA was equilibrated to a concentration of 20ng/µl using $T_{10}E_1$ (10 mM Tris; 1 mM EDTA, pH 8.0) buffer. For RAPD analysis each amplification mixture of 20 µl contained 30 ng genomic DNA, 2.5µl of 10× assay buffer (100 mM Tris.HCl, pH 8.3; 0.5 M KCl; 0.1% Gelatin), 2 mM $MgCl_2$, 200 µM each of the dNTPs, 20 ng of RAPD primers and 1 U *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). Amplification was carried out in a thermal cycler (GENEAMP-9700; Applied Biosystems, Foster City, USA) and it comprise an initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 60s, annealing at 37°C for 60s and an extension at 72 °C for 2 min, and final extension at 72 °C for 7 min. The amplified fragments were separated in 1.4 % agarose gel containing 0.5 ¼g ml^{-1} ethidium bromide in TAE buffer (40 mM Tris acetate, pH 8.0; 2 mM EDTA) at a constant 50 V for 60 to 80 min, and a tracking dye [20 % (w/v) sucrose; 0.1 M EDTA, 1.0 % (w/v) SDS; 0.25 % (w/v) bromo-phenol blue; 0.25 % (w/ v) xylene cyanol] was used to monitor the electrophoresis. The banding pattern was visualized under the gel documentation system (Geldoc XR system, Biorad, USA) and photographed. The sizes of fragments were calculated using 250 bp step up ladder (Bangalore Genei Pvt. Ltd.) as molecular weight marker, and TL120 software (Non-linear Dynamics, Total Lab Ltd., Newcastle Upon Tyne, UK).

Each amplified fragment was considered as unit character and was organized into 1-0 binary matrix, and Jaccards similarity coefficient was estimated. The pair wise similarity indices were used for the cluster analysis and generation of dendrogram using un-weighted pair group method with arithmetic mean (UPGMA), and also for principal coordinate analysis (PCoA; Mohammadi and Prasanna, 2003) using the NTSYSpc version 2.11s (Rohlf, 2008). The polymorphism information content (PIC) value for each locus was calculated using formula PICi = [2f_i (1" f_i)]/ n, where PICi is the polymorphic information content of the locus *i*, f_i is the frequency of the amplified fragments, (1" f_i) is the frequency of non-amplified fragments and 'n' represents total number of accessions (Roldan-Ruiz *et al.*, 2000). The PIC of each primer was calculated using the average PIC value from all loci of each primer. In addition average band informativeness (AvI_b) and resolving power (Rp) of RAPD primers were also estimated (Prevost and Wilkinson, 1999).

3. Results

3.1 Chromosome analysis and estimation of 4C DNA content

The root tip cells of *N. arbortristis* showed well condensed chromosomes at metaphase and somatic chromosome number was revealed to be 2n=44 (Fig. 1a). The 4C DNA content of all 14 ecotypes was quite homogeneous and varied from 18.94±0.82 to 21.46±1.88 pg (Table 1). The total chromosome length and TF% were also varied among the ecotypes from 98.7 to 102.7 µm and 39.8 to 44.08%, respectively (Table 1). Karyo-morphological studies revealed six (2n = 44: $2A_M + 26B_M + 8C_{SM} + 2D_{ST} + 4E_M + 2F_{SM}$) distinct chromosome types (Table 2, Fig. 1b). The karyotype of representative ecotype NAET-08 showed predominance of chromosomes with median (F% = 40.0% to 50.0%) and submedian (F% = 32.0% to 38.89%) centromere, though there were two sub terminal (F% = 19.04% to 22.72%) primary constrictions. Chromosome length was varied from 1.8 to 3.2 µm. Total chromatin length was 101.2 µm, and relative length of shortest chromosome arm compared to longest one (S%) was varied from 23.5 to 100.0% with average of 74.8%, and TF% was 102.09. Thus, the karyotype in *N. arbortristis* (NAET-08) was identified to be symmetric in nature and similar kinds of results were obtained for rest of the ecotypes tested here.

3.2 Meiotic analysis, pollen viability and germination of seeds

The pollen mother cells (PMCs) of 14 ecotypes revealed the gametic chromosome count is n = 22 (Fig 2a). Although in majority of PMCs across 14 ecotypes showed the existence of 22 normal bivalents (22II) as chromosome association at late diakinesis and metaphase-I, a few PMCs showed mixture of quadrivalents (IV), bivalents (II) and univalents (I) as chromosome associations (Table 3; Fig. 2b). Bivalents and univalents were observed in all ecotypes whereas quadrivalents were noticed in 10 ecotypes (Table 3). Mean value of quadrivalents, bivalents and univalent as chromosome association ranged between 0.36 to 1.6, 19.4 to 21.2 and 2.1 to 3.9, respectively (Table 3). Diplotene chromosomal configuration showed predominance of ring bivalents over rod bivalents with mean chiasmata of 23.95 in NAET02 ecotype to 24.43 in NAET 10 ecotype per cell (Range: 22-26). On average 22 chiasmata were terminalized with terminalization coefficient of 0.895 to 0.919 (Table 3). Majority of PMCs (80.1 to 88.6%) showed equal distribution (22II:22II) of chromosomes during anaphase-I (Fig, 2c), while rest showed unequal separation of chromosomes having complement 21II:2I:21II (10.2 to 16.4%) and 20II:4I:20II (1.3 to 5.4%) among the ecotypes assessed (Fig. 2d; Table 3).

In the present study, the pollen viability was ranged from 46.5% to 58.0% with an average of 52.39 among the studied ecotypes (Table 3). Pollens are spheroidal and lobate with smooth intine and unique exine having shrunken and tricolpate apertures which are appeared as granular pores. A germination assay of seeds of 14 ecotypes showed quite

low germination percentage as expected and was ranged between 42.33% in ecotype NAET 04 to 52.0 % in ecotype NAET 02.

3.2 Estimation of genetic variation among the ecotypes

Amplification with ten responding RAPD primers generated 60 unequivocal scorable DNA fragments, out of which 25 were polymorphic among the ecotypes tested (Fig 3). The range of amplified fragments varied from 365 to 3640 bp. A maximum of eight loci were amplified with primer OPA04 whereas, a minimum of four loci were amplified with the primer OPA05. These 10 primers were also exhibited a wide range of variation with regard to their PIC, AvIb and Rp (Table 4) with mean for the ten RAPD primers were 0.161, 1.673, and 10.327, respectively. The pairwise Jaccard's similarity coefficient values among the ecotypes were in the range of 0.648 to 0.962, which also evidenced low to moderate genetic variation among these ecotypes. NAET12 ecotype showed their closest affinity to NAET8 and NAET14 with similarity coefficient 0.962, whereas NAET4 and NAET5 ecotypes showed lowest similarity coefficient (0.648). The dendrogram generated by using the RAPD profile exhibited three major clusters in consonance with their geographical collection site baring one ecotype NAET10 (Fig. 4a). This clustering pattern was also affirmed by two dimensional principal coordinate analyses (PCoA; Fig. 4b), where two coordinates accounted for 57.05% of total genetic variation among the ecotypes of *N. arbortristis*.

4. Discussion

Medicinal plants possess unique identity in their 4C DNA content and somatic chromosome complements like other species, which could be evident in their size, shape and position of primary constrictions and also the secondary constriction and satellites, if any, in a few species. Studies on *in situ* nuclear DNA content along with karyotype analysis and behaviour of chromosomes during meiosis provide clues about the genome architecture in *N. arbortristis*.

In the present study, 14 ecotypes of *N. arbortristis* were assessed for somatic chromosome complement and 4C DNA content, and it has been observed that somatic chromosome count was 2n = 44 across all ecotypes with predominance of median and submedian chromosomes, thus symmetric karyotype. As expected, 4C DNA contents of all ecotypes reside in proximity of the mean values of 20.641pg. In addition to 2n= 44 (Bolkhorskikh *et al.*, 1969; George and Geethamma, 1984; George *et al.*, 1989), researchers have reported somatic chromosome numbers 2n = 46 (Kundu and De, 1968). This incongruence in somatic chromosome number might be attributed to the existence of different cytotypes in *N. arbortristis* in different geographical regimes as reported in *Abutilon indicum* (Bir and Sidhu, 1979; Krishnappa and Munirajappa, 1982; Rani *et al.*, 2012) and *Tribulus rajasthanensis* (Rawat *et al.*, 2006). The information generated from the present investigation, coupled with earlier published data, revealed that there are at least two somatic chromosome counts 2n = 2x= 44 and 2n= 2x=46 were adopted by the genotypes of *N. arbortristis* across different geographical regions of India including western Odisha. The 4C DNA content not varied significantly among the ecotypes tested in this study and this homogeneous distribution might be due to high G + C content of the genome and consistent occurrence of repetitive DNA sequences in the *N. arbortristis* genome as reported in other medicinal plants (Martel *et al.*, 1997; Behera *et al.*, 2010). The symmetric karyotype of *N. arbortristis* suggested towards its genetic stability across different habitats of a particular geographical regime (Schubert and Oud, 1997), and similar kind of reports were also made in *Cymbidium* (Sharma *et al.*, 2012), *Bacopa monnieri*, *Tylophora indica* and *Withania somnifera* (Samaddar *et al.*, 2012).

This somatic chromosome count was also reflected as chromosome associations in PMCs analysed at metaphase-I, where either 22 bivalents or a combination of quadrivalents, bivalents and univalents amounting into 44 chromosomes were noticed. Quadrivalents associations were occasionally noticed. The presence of quadrivalent indicate towards their evolutionary affinities among the chromosome(s) concerned and that they are formed by residual attractive forces acting between homeologous chromosomes as reported in *Brassica* sp. (Wills, 1966) and *Curcuma* sp. (Lamo and Rao, 2017). Even low frequency of quadrivalent associations as compared to bivalents has indicated towards allopolyploid lineage of *N. arbortristis* during the course of evolution and adaptation under different ecological niche (Feldman and Levy, 2005). The presence of univalents in the PMCs might be due to failure of synapsis, and the lowest mean value (2.1) of univalents was found in ecotype NAET12, while the maximum number of univalents per PMC (3.9) was recorded in ecotype NAET11. By and large, the univalents, wherever encountered, apparently behaved normally leading to an equal distribution of chromosomes at anaphase-I. However, in some cases these univalents were traced even at anaphase-I as laggards due to their non-disjunction towards respective poles. This kind of chromosomal behaviour might be due to desynapsis as reported in many tropical plants (Dawe, 1998; Rao and Kumar, 2003; Rawat *et al.*, 2006; Pradilo *et al.*, 2007; Behera *et al.*, 2010), and desynapsis among the bivalents might be attributed to influence of several environmental factors such as temperature, humidity and nutrition as reported in *Tribulus*

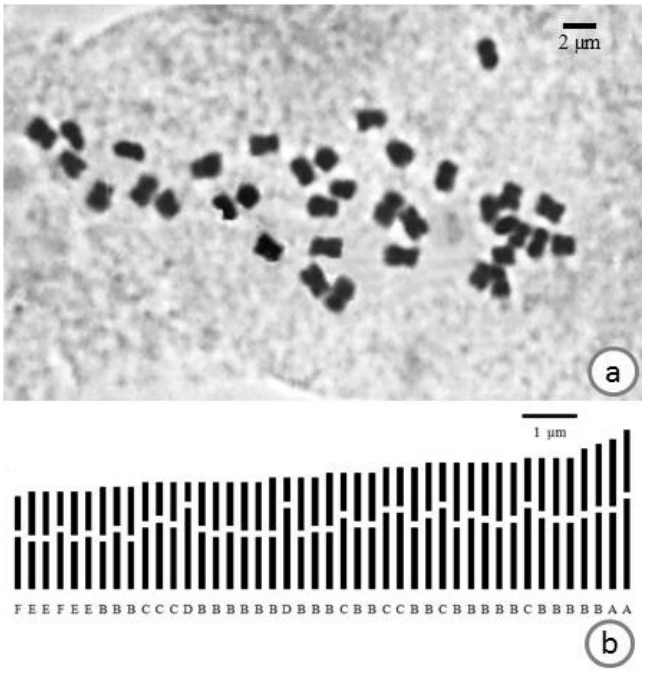

Fig.1: Mitotic analysis of *N. arbortristis*. (a) 2n=2x=44 chromosomes at early metaphase (Bar = 2.0 µM) and (b) Graphical representation of the symmetric karyotype of *N. arbortristis*, Ecotype NAET-08 (Bar = 1.0 µM).

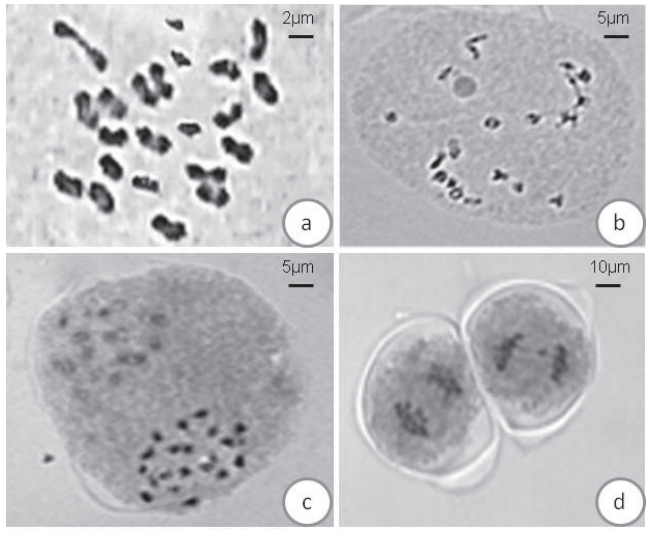

Fig.2: Meiotic behaviour of chromosomes in *N. arbortristis*. (a) Bivalents at late diakinesis metaphase; (b) Chromosome association (quadrivalents, bivalents and univalent) at metaphase-I; (c & d) Segregation of chromosome at anaphase-I with normal (c) and abnormal configurations showing laggards (d).

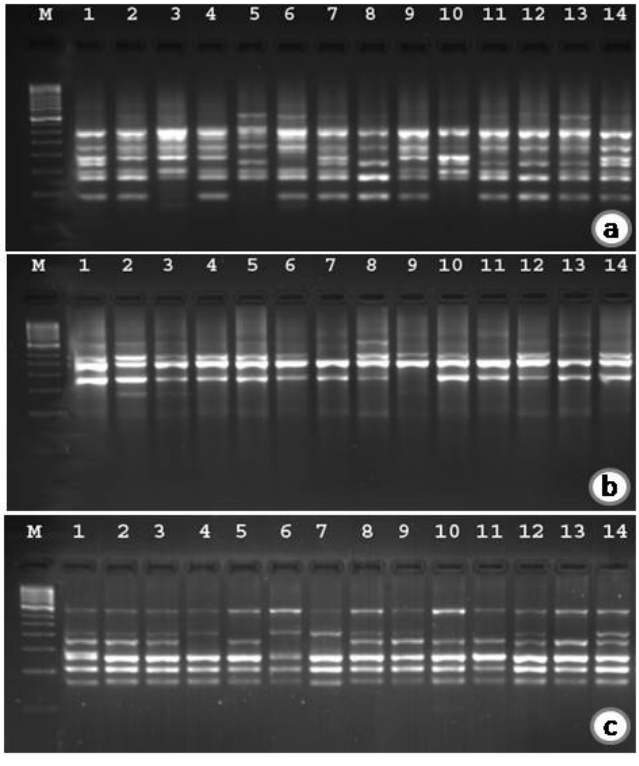

Fig.3: DNA banding pattern of 14 ecotypes of *N. arbortristis* generated by using RAPD primer OPA-04 (a), OPA-05 (b) and OPA-07 (c).

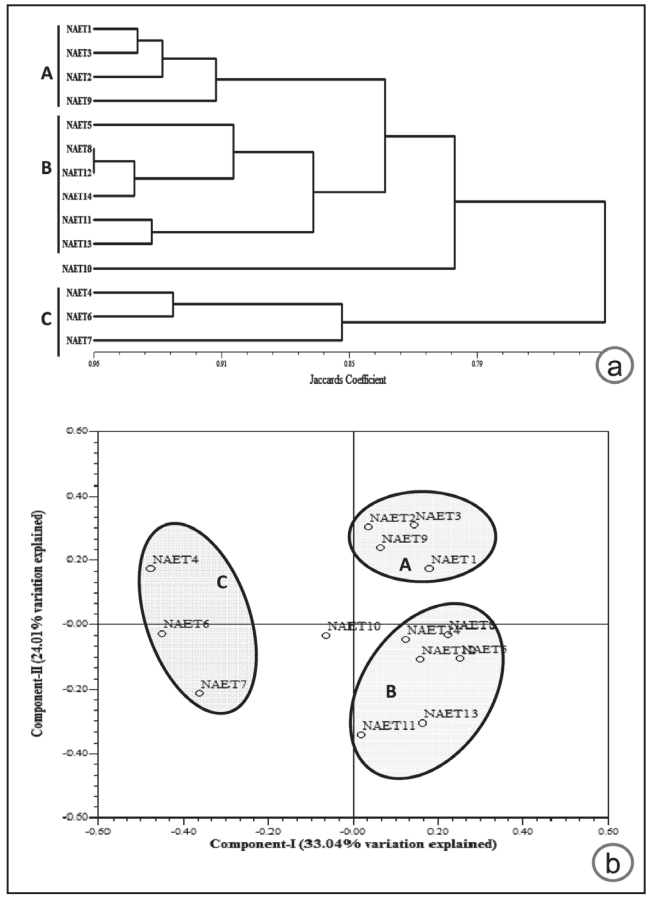

Fig.4: Genetic variation among 14 ecotypes of *N. arbortristis*. (a) Dendrogram (UPGMA) based on Jaccard's similarity coefficient and (b) Principal Coordinate analysis (PCoA) showing clustering of the ecotypes as depicted by RAPD marker analysis using 10 RAPD primers.

Table 1

Geographic distribution of 14 ecotypes collected from western Odisha, and their somatic chromosome count (2n), total chromosome length, TF% and 4C DNA content

Ecotype No.	Collection Site	Altitude (M)	Latitude (N)	Longitude (E)	2n	Total Chr. Length (μM)	TF%	4C DNA content (pg)
NAET1	Jharsuguda	218.0	21.8554°	84.0062°	44	99.90	44.01	20.48±2.48
NAET2	Jharsuguda	218.0	21.8554°	84.0062°	44	99.30	43.65	19.96±2.23
NAET3	Jharsuguda	218.0	21.8554°	84.0062°	44	100.60	43.01	20.86±2.11
NAET4	Sambalpur	135.0	21.4669°	83.9812°	44	100.20	42.65	20.90±2.22
NAET5	Bargarh	171.0	21.2550°	83.5070°	44	99.70	43.45	20.58±2.24
NAET6	Sambalpur	135.0	21.4669°	83.9812°	44	98.70	44.62	18.94±0.82
NAET7	Sambalpur	135.0	21.4669°	83.9812°	44	101.40	39.96	21.04±1.69
NAET8	Burla	173.0	21.4888°	83.8844°	44	101.20	42.09	21.02±1.68
NAET9	Jharsuguda	218.0	21.8554°	84.0062°	44	102.70	41.5	21.46±1.88
NAET10	Sambalpur	120.0	21.4669°	83.9812°	44	100.80	41.92	20.82±1.90
NAET11	Bargarh	171.0	21.2550°	83.5070°	44	101.00	40.8	20.94±1.82
NAET12	Burla	173.0	21.4888°	83.8844°	44	100.60	39.8	20.64±1.99
NAET13	Bargarh	171.0	21.2550°	83.5070°	44	100.50	44.08	20.50±2.22
NAET14	Burla	173.0	21.4888°	83.8844°	44	102.50	43.06	20.84±1.92
Mean					**44**	**100.65±1.07**	**42.47±1.47**	**20.641±1.94**

M: Meter; N: North; E: East; pg: picogram

Table 2

Karyotype details of *N. arbortristis* (Ecotype NAET-08) as revealed by its metaphase

Chromosome Type#	Number of chromosomes	Length* (μm)	Centromeric Index (F%)	Short Arm to long arm ratio (S%)	Centromeric position
A_M	02	3.0 - 3.2	40.625 – 46.667	0.684 – 0.875	Median
B_M	26	2.0 – 2.9	40.000 – 50.000	0.667 – 1.000	Median
C_{SM}	08	2.1 – 2.6	32.000 – 38.095	0.471 – 0.615	Sub-Median
D_{ST}	02	2.1 – 2.2	19.048 – 22.727	0.235 – 0.294	Sub-Terminal
E_M	04	1.9	42.105 – 47.368	0.727 – 0.900	Median
F_{SM}	02	1.8 – 1.9	36.842 – 38.889	0.583 – 0.636	Sub-Median
	44	1.8 – 3.2	19.048 – 50.000	0.235 – 1.000	—

*# A_M- Long median, B_M– Medium median, C_{SM}– Medium sub-median, D_{ST}– Medium sub-terminal, E_M– Shortmedian, and F_{SM}– Short sub median; * Length wise grouping: Short- < 2.0 μm; Medium- e"2.0 μm and < 3.0 μm; Long- e" 3.0 μm*

Table 3

Meiotic analysis in pollen mother cells (PMCs) of *N. arbor-tristis* at diplotene, diakinesis, metaphase-I and anaphase-I

Ecotype	Chromosome Association						Chiasmata properties				Terminalization coefficient	Anaphase-I Distribution (%)			Pollen viability (%age)	Seed germination Percentage (Mean ± SD)
	Quadrivalent (IV)		Bivalent (II)		Univalent (I)											
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	No. of Chiasmata Mean ± SD	Range	Unterminal-ised Chiasmata Mean ± SD	Terminalised Chiasmata Mean ± SD		22II:22II	21II:2I:21II	20II:4I:20II		
NAET01	0.45 ± 0.14	0-2	21.0±0.24	18-22	2.3 ±0.36	0-4	24.04±0.97	22-26	2.16±0.85	21.88±0.92	0.91	83.3	12.5	4.1	46.5	51.67±1.53
NAET02	0.85 ± 0.36	0-2	20.4±1.04	18-22	2.1±1.08	0-4	23.95±0.74	23-26	2.43±0.59	21.57±0.74	0.901	87.5	10.2	1.3	50.6	52.00±2.00
NAET03	0.75 ± 0.49	0-2	20.5±0.74	18-22	2.2±1.49	0-6	24.13±0.85	23-26	2.37±0.71	21.71±1.08	0.9	86.9	11.8	1.3	51.4	47.33±2.52
NAET04	—	—	20.8±0.65	19-22	3.4±1.36	0-6	24.11±0.87	23-25	2.47±0.69	21.58±0.83	0.895	80.2	14.5	5.4	53.4	42.33±1.53
NAET05	1.6 ± 0.24	0-2	19.5±0.93	18-22	2.8±1.92	0-4	24.13±0.87	23-26	2.21±0.73	21.87±0.69	0.906	80.1	16.2	3.7	51.2	47.67±0.58
NAET06	0.8 ± 0.64	0-2	19.4±1.03	18-22	3.2±2.06	0-4	23.95±0.80	23-25	2.33±0.79	21.57±0.97	0.901	89.5	10.2	0.3	58.0	52.00±1.00
NAET07	—	—	21.2±0.24	20-22	2.7±2.12	0-4	24.15±0.93	23-26	2.25±0.78	21.85±0.93	0.905	85.0	12.8	2.2	51.8	49.33±2.52
NAET08	—	—	20.3±1.03	18-22	3.8±2.06	0-6	24.1±0.85	23-25	2.20±0.76	21.85±0.83	0.907	81.8	16.4	1.7	50.8	45.67±0.58
NAET09	0.36 ± 0.14	0-2	20.5±0.93	19-22	2.7±1.86	0-4	24.0±0.97	23-25	2.05±0.78	22.0±0.66	0.915	85.0	14.2	0.8	54.2	50.33±1.53
NAET10	0.65 ± 0.36	0-2	19.5±0.93	18-22	2.8±1.87	0-4	24.43±0.81	23-26	2.43±0.59	21.95±0.74	0.899	84.2	13.2	2.6	53.6	51.00±1.00
NAET11	—	—	20.1±0.74	19-22	3.9±1.48	0-6	24.2±0.86	23-26	2.13±0.64	21.93±0.88	0.906	86.6	12.1	1.3	50.8	50.33±2.52
NAET12	0.75 ± 0.36	0-2	19.9±1.03	18-22	2.1±1.06	0-6	23.95±1.25	23-25	2.15±0.76	22.0±1.15	0.919	84.3	12.8	2.9	53.4	53.67±1.53
NAET13	1.6 ± 0.36	0-2	19.8±0.83	19-21	2.3±1.68	0-4	24.05±0.84	23-25	2.43±0.69	21.58±1.17	0.897	88.6	10.2	1.2	54.5	51.33±1.53
NAET14	1.1 ± 0.49	0-2	19.6±1.01	18-21	2.8±2.03	0-6	24.04±0.97	23-26	2.16±0.85	21.88±0.92	0.91	85.0	13.1	1.9	53.2	48.00±1.00

Table 4

Information about RAPD primers used for estimation of genetic diversity among 14 ecotypes of *N. arbortristis* showing polymorphic information content (PIC), average band informativeness (AvIb) and resolving power (Rp)

Primer	Primer sequence	No. of Loci amplified	Polymorphism (%)	Size amplified fragments (bp)	Polymorphic information content PIC	Average band Inform-ativeness (Av Ib)	Resolving power (Rp)
OPA 02	5'-TGCCGAGCTG-3'	5	40.00	585 - 1225	0.149	1.800	9.0
OPA 03	5'-AGTCAGCCAC-3'	6	50.00	480 - 1600	0.189	1.643	9.86
OPA 04	5'-AATCGGGCTG-3'	8	62.50	750 - 2165	0.253	1.607	12.86
OPA 05	5'-AGGGGTCTTG-3'	4	75.00	1190 - 2050	0.232	1.536	6.143
OPA 07	5'-GAAACGGGTG-3'	6	33.33	665 - 1905	0.124	1.833	11.0
OPA 08	5'-GTGACGTAGG-3'	9	44.44	365 - 2500	0.188	1.714	15.43
OPA 09	5'-GGGTAACGCC-3'	6	33.33	560 - 1925	0.145	1.786	10.71
OPA 13	5'-CAGCACCCAC-3'	5	20.00	455 - 1090	0.098	1.524	9.14
OPB 01	5'-GTTTCGCTCC-3'	5	20.00	580 - 3640	0.082	1.523	9.14
OPB 03	5'-CATCCCCCTG-3'	6	33.33	420 - 2595	0.153	1.764	9.99
	Total/ Average	60	41.66	365 - 3640	0.161	1.673	10.327

rajasthanensis (Rawat *et al.*, 2006). In majority of PMCs, the chiasmata were distally localized and were found to be terminalized by late diakinesis/early metaphase-I. This might be due to existence of short conserved segment of chromosomes and their involvement during the event of recombination, and such observations were commonly reported in species where the chromosome size is relatively small and morphological identical chromosomes are found (Dawe, 1998; Behera *et al.*, 2010). Presence of either distal or proximal chiasmata has been reported in several plant species (Gottschalk and Kaul, 1980; Kumar and Rao, 2002, 2003; Rawat *et al.*, 2006; Iqbal and Datta, 2007), and this has been attributed to chromosome pairing and/ or interference pattern coupled by availability of short segments for genetic crossing over in *N. arbortristis*. Majority of PMCs showed 22II:22II segregation of chromosomes, but in few PMCs laggards were observed during anaphasic separation. The presence of univalents and their subsequent failure in orientation and disjunction at anaphase might be associated with laggard formation (Gupta, 1995; Kumar and Rao, 2002; 2003; Rawat *et al.*, 2006; Iqbal and Datta, 2007; Sharma *et al.*, 2010).

The percentage of pollen viability was quite low, and it ranges from 46.5% to 58.0% across the ecotypes of *N. arbortristis* assessed, and it might be attributed to meiotic irregularities (Pagliarini, 2000) and environmental stress prevailed by relative humidity and temperature (Aronne, 1999). Similarly, the seeds showed very low germination rate (Rout *et al.*, 2008), which might be due to either abnormal pollen biology leading to unsuccessful fertilization event coupled with immature seed development or leaching out of phenolic compounds present in the pericarp and seed coat of *N. arbortristis* (Data not shown) as reported in other members of Oleaceae such as in *Abeliophylum* (Sahu *et al.*, 2012; Ghimire *et al.*, 2015).

Genetic polymorphisms among species and even between the genotypes of same species have manifold implications during the evolution and conservation of plant species across diverse ecological niche. Thus, depiction of genetic variation has long been based on morphological traits in general and even on phytochemical attributes in case of medicinal species. Both morphological and phytochemical attributes are subjected to penetrance and expressivity under the influence of environmental factors. Thus, diverse kinds of DNA markers have been used in recent time to assess the genetic variability as complimentary tools to conventional approaches in genetic resource management (Panigrahi *et al.*, 2015; Sahu *et al.*, 2016). However, the determinants of this variation have been poorly understood in *N. arbortristis*. Rohilla *et al.* (2017) used RAPD markers and reported on the existence of moderate genetic diversity among 16 accessions of Northern India. In the present study, 60 RAPD markers generated by ten responding primers substantiated the moderate genetic diversity among the 14 ecotypes *N. arbortristis* of western Odisha. In this study RAPD primers showed average PIC,

AvIb and Rp and estimation of moderate genetic divergence, and these findings were in good agreement with earlier report (Rohilla *et al.*, 2017). Dendrogram and PCoA based on Jaccard's similarity indices among these ecotypes of *N. arbortristis* formed three major clusters in consonance with their collection site. This might be due to similar kind of evolutionary forces at respective geographic regimes during the course of adaptation under different ecological niche. The similarity coefficient (0.648 to 0.962) was an indicator of moderate degree of genetic variation between *N. arbortristis* ecotypes used in this study. The ecotypes also showed their close genetic affinity among themselves and this might be due to their pedigree as reported in other medicinal species.

In summary, this study presented the suitability of RAPD markers as complimentary tool along with conventional cytological analysis for genetic divergence study in *N. arbortristis*. Use of advanced generation DNA markers such as simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) could also be used as genomic tools for elucidation of genetic relationships and domestication pathway of this medicinal species, and genetic augmentation of *N. arbortritis* through molecular breeding aiming at superior therapeutic potential.

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