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Characterization of intraspecific hybrid in *Clitoria ternatea* (L.) using morpho-physiological, cytogenetic, metabolic and molecular markers

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Abstract. *Clitoria ternatea* (L.) is a medicinal plant possessed with bioactive molecules such as taraxerol, delphinidin, kaempoferol and quercetin etc. For the development genotype with higher content of these bioactive molecules, marker-assisted breeding is one of the best strategies and it initiates with the development of F_1 hybrids. Thus, an intraspecific F_1 hybrid was raised involving two contrasting genotypes of *C. ternatea acc. CtB3-SL1* (Blue flowered) and *C. ternatea acc. CtW2-BL1* (white flowered). The hybridity of the F_1 plant was confirmed by assessing the phenotypic traits, such as colour of the petal, pod shape and seed coat colour, 100 seed weight, and the content of taraxerol and delphinidin. The pollen mother cells in the F_1 hybrid showed eight bivalents with preponderance of ring bivalents and 8I:8I segregation at metaphase-I and Anaphase-I, respectively. SDS-PAGE seed albumin and globulin detected three pollen parent-specific polypeptides (Mw 31.62, 22.38 and 18.81KDa), and were inherited to F_1 hybrids, which evidenced the hybridity of putative F_1 plants. Further, DNA marker analysis also showed the inheritance of 11 RAPD, six SCoT and one ISSR markers to putative F_1 plant, which affirmed the hybrid nature of the F_1 plant. This study also evidenced that combined use of morphophysiological, cytogenetic, protein and DNA marker analyses could be effective for precise characterization of intra-specific hybrids in *C. ternatea*. These F_1 hybrid and its derived future progenies could also be used for mapping of QTLs or genes contributing higher accumulation of taraxerol and delphinidin in different plant parts.

Keywords. Hybridity, Meiosis, Seed protein profile, DNA marker, Taraxerol, Delphinidin.

INTRODUCTION

Clitoria ternatea (L.) belongs to the family-Leguminosae (Fabaceae) with somatic chromosome count 2n=2x=16 and was distributed worldwide

(Joson and Ramirez 1991; NPGS2008). The *C. ternatea* is also known as Shankhpushpi, Butterfly pea, Aparajita, Gokarni, Girikarnika (Bishoyi et al. 2014). The plant is elongated, slender, climbing herbaceous vine with five leaflets, white to purple flowers, and has deep roots. Butterfly pea is predominantly a self-pollinated species, but sometimes considered as often cross-pollinated due to the appearance of segregating genotypes in some populations (Cook et al. 2005). This plant has been considered as important medicinal plant, and the phyto-chemical studies explored several bioactive metabolites such as alkaloids, triterpenoids, flavonoids, glycosides, anthocyanins and lactones etc. in this species (Mukherjee et al. 2008; Sethiya et al. 2009). Thus the plant parts based extracts have been prepared and used as a memory enhancer, nootropic, anti-stress, anxiolytic, anticonvulsant, tranquilizing and sedative agent (Parrotta 2001; Prajapati et al. 2003; Khare 2004; Kapoor 2005; Margret et al. 2015). Among the bioactive metabolites, content of kaempferol, delphinidin and taraxerol having anti-cancer and anti-tumour properties have also been documented in this species (Braig et al. 2005; Chen and Kong 2005; Niering et al. 2005; Singletary et al. 2007; Swain et al. 2012). The white-flowered genotypes of *C. ternatea* were found to be medicinally rich in taraxerol and kaempferol, whereas the blue flowered genotypes were affluent in delphinidin, quercetin and isoquercetin etc. For the development of improved genotype with higher content of these bioactive metabolites marker assisted breeding (MAB) is one of the finest strategy, where F_1 hybrid serves as the starting material for all future breeding efforts for the genetic improvement. Thus intra-specific F_1 hybrid involving blue flowered and white flowered genotype can be used as the starting material for widening genetic base of this medicinal species in term of bioactive metabolites composition and production of advanced breeding lines containing desired metabolites of pharmaceutical importance. However, interaction between both the genome in either of the parental cytoplasmic background often led to genetic variation due to genetic recombination, differential gene action, penetrance and expressivity. F_1 hybrid being the starting material for all breeding efforts, its precise identification at early stage is mandatory. Thus morphological, cytological, biochemical and molecular markers have been effectively used to ascertain hybridity of F_1 plants in many species. But the reproducibility of morphological, cytological and biochemical markers in consonance to environmental variation and developmental regulations limits their applicability. Therefore seed protein and DNA sequence based marker analysis could also be utilized to screen and identify F_1 hybrids at an early stage because of their stability, uniformity, reliability and reproducibility across the environment and are also free from penetrance and expressivity.

The seed protein markers have been effectively employed for cultivar characterization, genetic diversity assessment, and verification of hybridity in many species (Mohanty et al. 2001; Panigrahi et al. 2007; Jisha et al. 2011; Mishra et al. 2012). Similarly DNA markers, such RAPD, ISSR and SSR have been used for characterization of hybrids in different species (Lima-Brito et al. 2006; Muthusamy et al. 2008; Goldmann et al. 2008; Hemalatha et al. 2010; Bianco et al. 2011; Mishra et al. 2012; Mishra et al. 2017). In case of *C. ternatea*, few of these DNA markers have only been used for genetic diversity studies (Chandra 2011; Swati et al. 2011; Ganie et al. 2012; Ali et al. 2013: Bishoyi et al. 2014). However, no report has been made so far on the development of intra-specific hybrid in *C. ternatea* and its characterization.

In the present study, an intra-specific F_1 hybrid of *C. ternatea*, involving blue flowered [*C. ternatea acc. CtB3-SL1*] and white flowered [*C. ternatea acc. CtW2-BL1*] genotype, was raised and its hybridity was affirmed by simultaneous use of morpho-physiological and cytogenetic analyses, estimation of bioactive metabolite, profiling of seed protein and DNA based markers.

MATERIALS AND METHODS

Plant materials

Genotypes of *C. ternatea* (including of five white genotypes, six blue genotypes, and four bipetaloid blue genotypes) were collected from Sambalpur and Bargarh districts of Odisha and maintained at the experimental garden, School of Life Sciences, Sambalpur University, Odisha, India (Table 1). Among these genotypes, two genotypes of *C. ternatea acc. CtB3-SL1* and *C. ternatea acc. CtW2-BL1* were identified on the basis of their bioactive metabolites (Delphinidin and Taraxerol) content, and used as seed parent and pollen parent, respectively for the development of F_1 hybrid.

Morpho-physiological traits characterization

Different morpho-physiological traits like colour of standard petal, flower length, flower breadth, floral bud size, anther size, style length, stigma length, seed coat colour and 100 seeds weight were studied for three F_1 plants along with their parents. The morpho-physiological traits unique to pollen parent were used as visual DUS marker for the characterization of F_1 hybrid.

Table 1. Fourteen accessions of *C. ternatea* with their flower colour, petal configuration and the geographical coordinates of collection sites located in Odisha, India.

Sl. no.	Accession (acc._)	Collection Site	Latitude	Longitude	Mean Sea Level(m)	Flower Colour & Petal structure
1	CtW1-BG1	Bargarh	21°22'50"N	83°44'48"E	186	White unipetaloid
2	**CtW2-BL1**	**Sriram vihar (Burla)**	**21°28'46"N**	**83°53'5"E**	**172**	**White unipetaloid**
3	CtW3-BL2	Burla Town	21°28'46"N	83°53'5"E	172	White unipetaloid
4	CtW4-BGK1	Kandahata (Bargarh)	21°15'57"N	83°39'54"E	186	White unipetaloid
5	CtW5-BG2	Bargarh	21°22'50"N	21°22'50"E	186	White unipetaloid
6	CtW6-BG3	Bargarh	21°22'50"N	21°22'50"E	186	White unipetaloid
7	CtB1-KL1	kuchinda	21°37'34"N	83°19'0"E	254	Blue unipetaloid
8	CtB2-BGK2	Kandahata (Bargarh)	21°15'57"N	83°39'48"E	186	Blue unipetaloid
9	**CtB3-SL1**	**Sambalpur**	**21°46'81"N**	**83°97'54"E**	**151**	**Blue unipetaloid**
10	CtB4-PL1	Padampur (Bargarh)	21°0'0"N	83°3'46"E	205	Blue unipetaloid
11	CtB5-PL2	Padampur (Bargarh)	21°0'0"N	83°3'46"E	205	Blue unipetaloid
12	CtBB1-PL3	Padampur (Bargarh)	21°0'0"N	83°3'46"E	205	Blue bi-petaloid
13	CtBB-PL4	Padampur (Bargarh)	21°0'0"N	83°3'46"E	205	Blue bi-petaloid
14	CtBB-PL5	Padampur (Bargarh)	21°0'0"N	83°3'46"E	205	Blue bi-petaloid

Cyto-genetic characterization

To study the chromosome homology, mitotic and meiotic analysis of F_1hybrids and their parents were carried out following Behera et al. (2010). Well-developed roots (1-3 cm) obtained from the seedlings at 8.00-8.30 a.m. and incubated with pre-chilled p-dichlorobenzene (PDB) solution for two hours at 20°C, 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 was 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. For meiotic analysis, the flower buds of appropriate size were fixed in 1:3 aceto-alcohol and kept overnight at 25 ± 2°C and then it was transferred to 70% ethanol and stored at 4°C. The anthers of suitable size were squashed in a drop of 1.5% acetocarmine, and the meiotic behaviour of chromosomes at diakinesis, metaphase-I and anaphase-I were observed. Suitable stages of mitosis and meiosis were observed under a compound microscope (Unilab, India) and were documented using Nikon Coolpix-4500 camera.

Bioactive metabolites characterization

Estimation of taraxerol in root tissues: Roots of *C. ternatea* genotypes and their F_1 hybrid were collected after 30 days of initiation of flowering, air dried under shade and ground to fine powder. Powder of each sample (appx. 20gm) was subjected to extraction with 70% alcohol for 5h at 60°C, filtered (using Whatmann No.1 filter paper) and dried under vacuum in a rotary evaporator (RV-10, IKA, Germany). The hydroalcoholic extract (appx. 8.4 g) was suspended in water and sequentially extracted using hexane, chloroform, ethyl acetate and n-butanol as described by Kumar et al. (2008). The hexane and chloroform fractions were subjected to chromatography using Chlorofom: methanol (1:1, v/v) as eluent and the eluted fractions were further chromtographed using hexane: ethyl acetate (80:20, v/v) as eluent and yielded the delphinidin as described earlier (Kumar et al. 2008). This compound was dissolved in ethanol (1mg.ml^{-1}) and 10 µl aliquot of each sample was used for HPTLC assay along with standard taraxerol solution (10-100µg.ml^{-1}) as described by Kumar et al. (2008). Thin layer chromatography was carried out using aluminum backed HPTLC plates (100cm^2; 0.2 mm thickness) of silica gel 60 F_{254} (Merck, Germany) in a HPTLC system (CAMAG, Switzerland) consisting of Linomat-IV sampler, twin plate development chamber and CAMAG TLC scanner 3 with WINCATS software. The derivatized plates were scanned under visible light and the content of taraxerol was estimated densitometrically by measuring absorption at 420 nm by TLC scanner-3 integrated with WINCATS v 1.4.2 software (slit dimension- 6 mm x 0.45 mm; scanning speed- 20 mm.s^{-1}).

Estimation of delphinidin in flowers: The flowers were collected, air dried and extracts of petals were prepared following Fukui et al. (2003). The petal

extracts were dissolved in 0.2 ml of 6 N HCl and kept at 100 °C for 20 min. The hydrolyzed anthocyanidins were extracted with 0.2 ml of 1-pentanol. HPLC was performed using an ODS-A312 column as described by Katsumoto et al. (2007) using acetic acid : methanol : water (15 : 20 : 65) as solvent with flow rate 1ml per min, and the delphinidin content was estimated by measuring absorbance from 400-600 nm on photodiode array detector (SPD-M10A; Shimadzu Co., Ltd). Under these HPLC conditions, the λ_{max} of delphinidin and retention time were 540 nm and 4 min, respectively which were validated with those of delphinidin chloride (Sigma-Aldrich).

Proteomic characterization using seed protein profiling

The albumin and globulin fraction of the seeds were extracted and denatured as described by Panigrahi et al. (2007). Protein samples (appx. 25.0 µg) were separated under discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli et al. 1970) using 10 % resolving gel (0.375 M Tris–HCl, pH 8.8) and 4% stacking gel (0.125 M Tris–HCl, pH 6.8). Tris-glycine (0.1% SDS, 25 mM Tris-glycine, pH 8.3) was used as running buffer, and electrophoresis was carried out at 1.5 mA per well constant current until tracking dye reaches the separating gel, and then current supply was increased to 2 mA per well till tracking dye reach bottom of the gel. The molecular weight marker-PMWM (Genei Pvt. Ltd.) was used as a standard, and the size of polypeptides was estimated by standard curve method.

Genomic characterization using DNA markers

The genomic DNA from F_1 hybrid and its parents were isolated using the modified CTAB method (Sivaramakrishnan et al. 1997) and purified (Mishra et al. 2012). DNA was dissolved in 2.0 ml TE (Tris-EDTA) buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at -20°C. Purity and concentration of the DNA sample were measured using a UV-Vis spectrophotometer (UV 1601, Shimadzu, Kyoto, Japan) by taking TE buffer as the blank. The quantification of DNA was validated by analyzing the purified DNA on 0.8 % (w/v) agarose gel by taking diluted, uncut phage lambda DNA as standard. The DNA samples were equilibrated to 10 ngµl^{-1} in TE buffer. For RAPD, SCoT and ISSR marker analysis, the PCR amplification of 25ng of genomic DNA was carried out using 30 random decamer oligonucleotide primers (OPA-01-20 and OPB-01-10; Operon Technologies, Alameda, CA, USA), 36 SCoT primers (SCoT -1 to SCoT -36; Collard and Mackill 2009) and seven ISSR primers from the set 100/9 (UBC-861, UBC-865, UBC-868, UBC-873, UBC-872, UBC-808, UBC-807; University of British Columbia, Vancouver, Canada), respectively. The PCR amplification reaction (25µl) contained 25 ng template DNA, 2.5µl of 10X assay buffer [100 mM Tris-Cl, pH 8.3; 0.5 M KCl; 0.1 % (w/v) gelatin], 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.25µM primer, 1.0 units *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). For RAPD, ISSR and SCoT analysis, the amplification were carried out using a thermal cycler (GENEAMP-9700; Applied Biosystems, Foster City, USA), and conditions are as below:

Amplification Conditions	RAPD	ISSR	SCoT
Initial Denaturation	94 °C for 5 min		
PCR cycles	45 cycles	40 cycles	35 cycles
Cyclic Denaturation	94 °C for 60 s	94 °C for 30 s	94 °C for 30 s
Annealing of primers	37 °C for 60 s	40-60 °C for 60 s	50 °C for 60 s
Elongation	72 °C for 2 min	72 °C for 2 min	72 °C for 2 min
Final Elongation	72 °C for 5 min	72 °C for 5 min	72 °C for 5 min
Storage of sample	4 °C for ∞ min		

The amplified products were mixed with gel loading buffer [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] and separated in 1.4 % (w/v) agarose gel containing 0.5µgml^{-1} ethidium bromide in TAE buffer (40 mM Tris acetate, pH 8.0; 2 mM EDTA) at 50V constantly. The separated DNA fragments were documented using gel documentation system (Gel Doc XR system, Biorad, USA), size of amplified fragments was estimated using TL-120 software (Non-linear Dynamics, Total Lab Ltd., Newcastle Upon Tyne, UK) and 250 bp step-up ladder (Bangalore Genei Pvt. Ltd.) as standard.

RESULTS

In this study, the putative intra-specific F_1 hybrids were raised by convetional hybridization and were characterized by using morpho-physiological and cytogenetic analysis, estimation of bioactive metabolites, seed protein (albumin and globulin) profiling and DNA marker analysis.

Morpho-physiological and metabolite characterization of the intraspecific F_1 hybrid

Morpho-physiological traits including flower colour, pod beak, and seed coat colour distinguish the blue flowered parental genotype *C. ternatea* (*acc. CtB3-SL1*) from white flowered one (*C. ternatea acc. CtW2-BL1*). The size of flower and 100 seed weight were also distinguishes both the parents. In many such morpho-physiological traits, the F_1 hybrid was intermediate between the parents with predominance of the characters of *C. ternatea*, *acc. CtW2-BL1*, such as seed coat colour, petal colour being the pollen parent, and their appearance in the intermediate form was also very vivid for the identification of the F_1 hybrid (Table 2; Fig. 1a, b). In most of the quantitative traits, such as leaf size, flower size, and 100 seed weight, the F_1 plant resided well around the mid-parental value (Table 2).

The raised F_1 hybrids were assessed along with their parents for two important bioactive metabolites (Taraxerol and Delphinidin). Taraxerol was obtained mainly from root tissues whereas delphinidin was obtained from the petals of the flowers. The F_1 hybrid contains 0.856±0.031 mg.g^{-1} taraxerol in its root tissue and 0.372±0.019 mg.g^{-1} delphinidin in its flowers. On comparison with its parents taraxerol content in root tissue of F_1 hybrid was almost at par with the donor parent (*C. ternatea acc. CtW2-BL1*) whereas delphinidin content was intermediate between both the parents (Table 2; Fig. 1c).

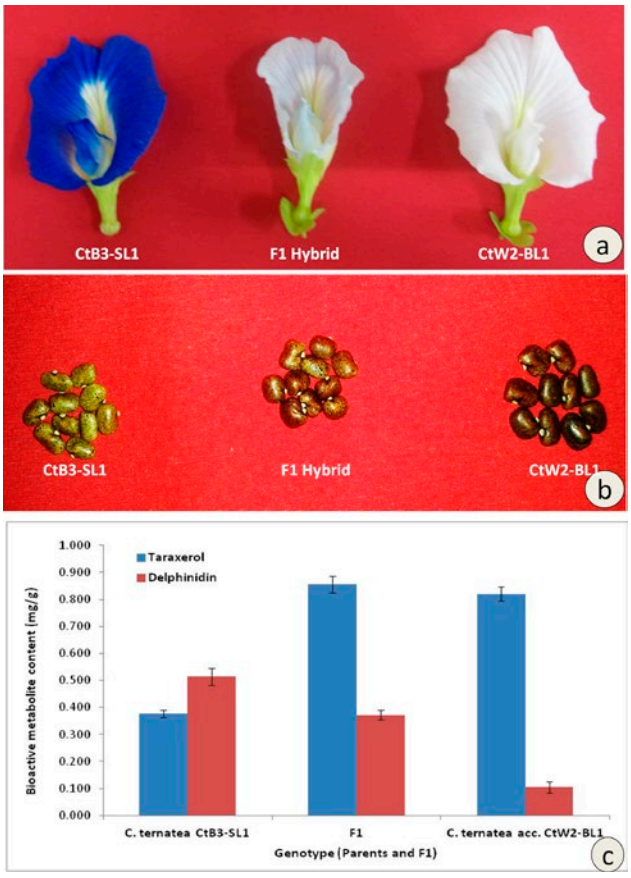

Fig. 1. Characterization of intra-specific F_1 hybrid along with its parents (*C. ternatea acc. CtB3-SL1* and *C. ternatea acc. CtW2-BL1*). Floral attributes including petal colour (a), Mature seeds showing colour of the seed coat and aril (b); Graphical representation of bioactive metabolites (taraxerol and delphinidin) content.

Cyto-genetical characterization of the intraspecific F_1 hybrid

Appropriate stages like metaphase, anaphase, diakinesis, metaphase-I, anaphase-I were observed in the

Table 2. Morpho-physiological and metabolite characterization of *intra-specific F_1* hybrid of *C. ternatea.*

Morphological traits	*C. ternatea acc. CtW2-BL1*	*C. ternatea acc. CtB3-SL1*	F_1 hybrid
Shape of the leaflets	Lanceolate	Lanceolate	Ovate-lanceolate
Base of the leaflets	Cuneate	Oblique	Oblique
Flower length (cm)	5.23±0.15	5.03±0.15	5.08±0.1
Flower breadth (cm)	3.2±0.1	3.04±0.06	3.07±0.06
Average days to flowering	56 days	64 days	64 days
Colour of the Petal	White	Blue	Intermediate
Nature of the ovary & style	Pubescent	Glabrous	Glabrescent
Seed colour	Black	Brown	Blakish brown
Number of seeds per pod	4-5	5-6	4-5
100 Seed weight (g)	5.82 ± 0.19	5.16 ± 0.2	5.26 ± 0.18
Taraxerol Content (mg.g^{-1})	0.821 ± 0.026	0.377 ± 0.014	0.856 ± 0.031
Delphinidin Content (mg.g^{-1})	0.104 ± 0.02	0.514 ± 0.019	0.372 ± 0.019

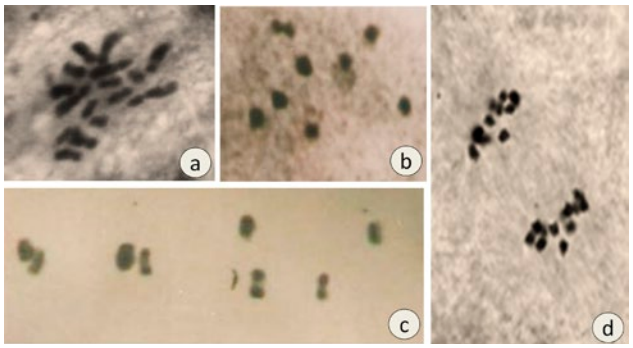

Fig. 2. Cytogenetic characterization of F_1 hybrid showing 2n=2x=16 chromosome configuration at mitotic metaphase (a), association of eight bivalents at diakinesis (b) and metaphase-I (c), and segregation of chromosome at anaphase-I (d).

PMCs of both the parents and F_1 hybrid. Chromosome analysis of both the parents revealed 16 chromosomes at metaphase in each of them, and the separation at anaphase occurs in a normal fashion. The mitotic metaphase of F_1 hybrid showed 16 distinct chromosomes similar to its parents (Fig. 2a). As expected, the PMCs of the F_1 hybrid showed formation of eight bivalents (II) at diakinesis and metaphase-I (Fig. 2b, c) and 8II: 8II separation at anaphase-I (Fig. 2d). The pollen fertility in the F_1 hybrid was almost 86% and was equivalent to its parents.

Proteomic and genomic characterization of the intraspecific F_1 hybrid

SDS-PAGE of seed albumins of two parental genotypes, including *C. ternatea acc. CtB3-SL1* and *acc. CtW2-BL1,* and their F_1 hybrids led to the detection of 33 polypeptide bands with molecular weight 12.59 to 84.14 KDa**.** Out of which 30 polypeptides were monomorphic, and rest three were varied for their expression (Table 3; Fig. 3). The putative F_1 hybrid possessed with three polymorphic polypeptides (Mw 31.62, 22.38 and 18.81 KDa) specific to pollen parent *C. ternatea acc. CtW2-BL1* along with the monomorphic polypeptides (Table 3; Fig. 3). Since *C. ternatea acc. CtW2-BL1* was used as pollen parent, the appearance of these unique albumin polypeptides in the F_1 hybrid can potentially be used as markers for identification of hybrids involving at least *C. ternatea acc. CtW2-BL1* as pollen parent.

In the present study, all three kinds of DNA markers showed polymorphism *at par.* Thirty RAPD primers have amplified 127 RAPD fragments ranging from 130 to 2389 bp, and among them, 22 amplified fragments (17.32%) showed parental polymorphism (Table 4). Contrasting to this amplification with nine ISSR and

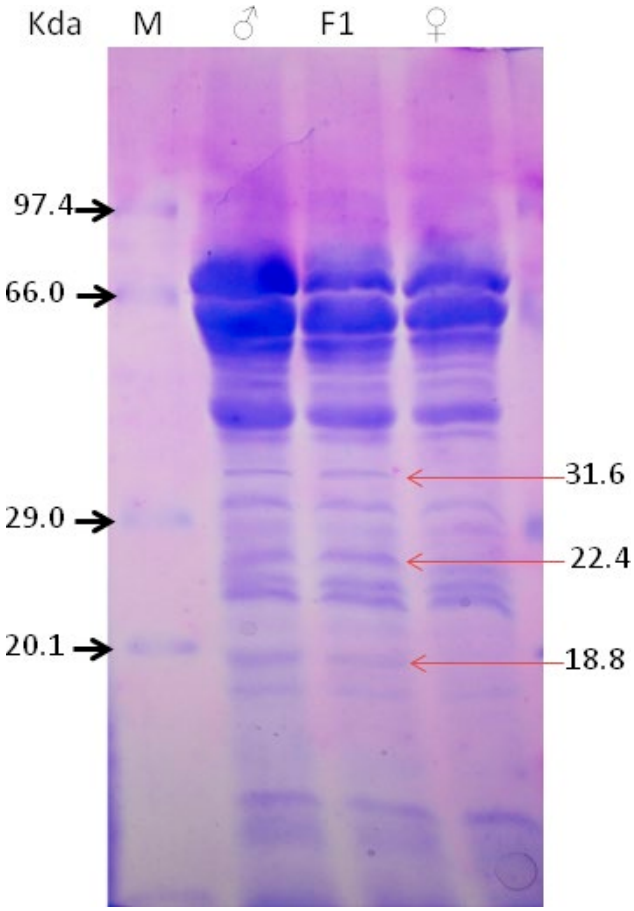

Fig. 3. Inheritance of seed albumin markers to intra-specific F_1 hybrid (*C. ternatea acc. CtB3-SL1* X *C. ternatea acc. CtW2-BL1*) intra-specific F_1 hybrid; Lane 'M' represents Mol. Weight Marker (PMW-M, GENEI, India), and arrow indicates on right hand side indicate the polypeptides inherited to the F_1 hybrid (MW in kDa).

36 SCoT primers generated 39 and 224 fragments ranging from 437 to 3154 bp and 116 to 3916 bp, respectively (Table 4, 5). Both ISSR and SCoT analysis showed lower polymorphism (2.56% and 5.80%) in comparison to RAPD markers. Among the parental polymorphic markers 22 RAPD, one ISSR and 13 SCoT markers were inherited to the putative F_1 hybrid, and among them 11 RAPD and six SCoT markers, unique to pollen parent (*CtW2-BL1*), were very vivid in its appearance for the identification of F_1 hybrid (Fig. 4). The total number of fragments amplified, percentage of polymorphism, inheritance of polymorphic fragments to the F_1 hybrid were shown in Table 4 and 5**.**

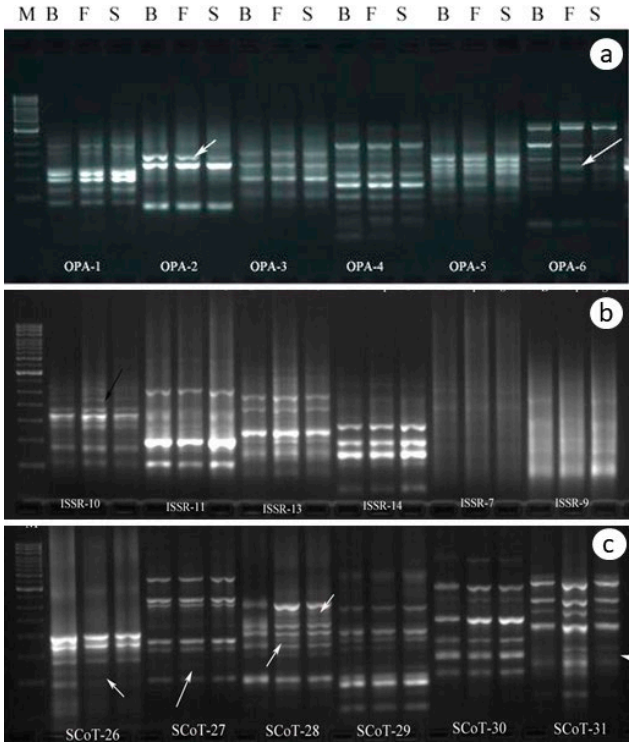

Fig. 4. Inheritance of pollen parent specific fragments (→) to *C. ternatea acc. CtB3-SL1* X *C. ternatea acc. CtW2-BL1* intra-specific F_1 hybrid, generated by RAPD primers (a), ISSR primers (b) and SCoT primers (c). (M: 250 bp step-up ladder, B: *C. ternatea acc. CtW2-BL1*, F: F_1 hybrid and S: *C. ternatea acc. CtB3-SL1*).

DISCUSSION

The F_1 hybrids have been considered as starting material for all breeding endeavours including the development of mapping population, mapping, and tagging of traits, marker-aided selection and generation of advanced breeding lines before the release of cultivars. Thus, identification and characterization of F_1 hybrids at an early stage during hybridization programme is quite essential (Lima-Brito et al. 2006; Mishra et al. 2012). In this study, the putative intra-specific F_1 hybrid was characterized by using morpho-physiological and cytogenetic analyses, estimation of bioactive metabolites (taraxerol and delphinidin), seed protein (albumin and globulin) profiling, and DNA marker analysis.

The F_1 hybrid was intermediate between the parents (*C. ternatea acc. CtB3-SL1* and *acc. CtW2-BL1*),in term of morpho-physiological traits distinguish the parents, such as flower colour, pod beak and seed coat color, size of flower and 100 seed weight. Although no pervious report is available in *C. ternatea*, in several species the character(s) distinguish the parents were appeared in its intermediate form in F_1 hybrid (Mishra et al. 2012; Mishra et al. 2017) and in some cases the pollen parent specific traits were also predominated as observed in the present study. Either the appearance of pollen parent specific traits in the F_1 or appearance of traits in intermediate form could be used vividly for the identification of hybridity. The consistency of metabolites in the F_1 hybrid of any medicinal plant in consonance to their parents is very vital in the perspective of their use as source material either to harvest therapeutics compounds or to generate breeding lines. *C. ternatea* plant parts are source of many important bioactive metabolites, and also have wide range of biological and pharmacological activities (Mukherjee et al. 2008; Sethiya et al. 2009). In view of this, the raised F_1 plants were assessed along with their parents for two important bioactive metabolites, Taraxerol and Delphinidin, mostly used for the treatment of various kind cancer and tumours (Braig et al. 2005; Chen and Kong 2005; Niering et al. 2005; Singletary et al. 2007; Swain et al. 2012). Taraxerol was obtained mainly from root tissues whereas delphinidin was obtained from the petals of the flowers. The F_1 hybrid contains 0.856±0.031 mg/g Taraxerol in its root tissue and 0.372±0.019 mg/g delphinidin in its flowers. On comparison with its parents taraxerol content in root tissue of F_1 hybrid was almost at par with the pollen parent (0.821±0.026) whereas delphinidin content was intermediate between both the parents (*C. ternatea acc.*

Table 3. Details of seed albumin and seed globulin markers inherited to the intraspecific F_1 hybrid.

Marker	Total no of bands	Range of Molecular Weight (KDa)	No. of polymorphic polypeptides	No. of polymorphic bands inherited to F_1 from		Parents specific bands in F_1 from (kDa)	
				CtW2-BL1	*CtB3-SL1*	*CtW2-BL1*	*CtB3-SL1*
Seed albumin	20	14.13-66.83	03 (15.0%)	03	--	**31.6, 22.4 & 18.8**	--
Seed globulin	13	12.59-84.14	--	--	--	--	--
Total	**33**	**13.34-112.2**	**03 (9.09%)**	**03**	**--**	**31.6, 22.4 & 18.8**	

Table 4. Details of RAPD and ISSR markers used for characterization of intraspecific F_1 hybrid showing the inheritance of parent specific markers to the intraspecific F_1 hybrid.

Primer	Sequence (5 ' → 3')	No. of fragments amplified	Range of amplified fragments (bp)	Polymorphic bands	Percentage of polymor-phism (%)	No. of Non-parental fragments* in F_1	Parent specific polymorphic bands (bp) in F_1 from	
							CtW2-BL1	*CtB3-SL1*
RAPD Marker								
OPA-01	CAGGCCCTTC	7	176-1500	---	--	$OPA1_{1500}$	---	---
OPA-02	TGCCGAGCTG	3	432-1233	$OPA-02_{1233}$	33.33	---	---	$OPA-02_{1233}$
OPA-03	AGTCAGCCAC	4	273-968	---	0	---	---	---
OPA-04	AATCGGGCTG	9	170-1500	$OPA-04_{170}$ $OPA-04_{314}$	22.22	---	$OPA-04_{170}$	$OPA-04_{314}$
OPA-05	AGGGGTCTTG	6	506-1233	$OPA-05_{506}$	16.66	---	---	$OPA-05_{506}$
OPA-06	GGTCCCTGAC	8	276-2045	$OPA-06_{1516}$ $OPA-06_{612}$	25	---	---	$OPA-06_{1516}$ $OPA-06_{612}$
OPA-07	GAAACGGGTG	5	530-1937	---	---	---	---	---
OPA-08	GTGACGTAGG	4	130-1019	$OPA-07_{895}$	25	---	$OPA-07_{895}$	---
OPA-09	GGGTAACGCC	5	461-1401	---	---	---	---	---
OPA-10	GTGATCGCAG	2	277-911	---	---	---	---	---
OPA-11	CAATCGCCGT	6	139-2389	$OPA-11_{1079}$	16.66	---	$OPA-11_{1079}$	---
OPA-12	TCGGCGATAG	5	330-2333	$OPA-12_{2333}$	20	---	$OPA-12_{2333}$	---
OPA-13	CAGCACCCAC	3	758-1144	---	---	---	---	---
OPA-14	TCTGTGCTGG	2	599-717	---	---	---	---	---
OPA-15	TTCCGAACCC	9	284-2250	$OPA-15_{2250}$ $OPA-15_{622}$	22.22	---	$OPA-15_{2250}$ $OPA-15_{622}$	---
OPA-16	AGCCAGCGAA	9	299-2187	$OPA-16_{2167}$ $OPA-16_{622}$	22.22	---	$OPA-16_{2167}$	$OPA-16_{622}$
OPA-17	GACCGCTTGT	1	437	---	0	---	---	---
OPA-18	AGGTGACCGT	2	569-974	$OPA-18_{974}$	50	---	$OPA-18_{974}$	---
OPA-19	CAAACGTCGG	1	1193	$OPA-19_{1193}$	100	---	---	$OPA-19_{1193}$
OPA-20	GTTGCGATCC	1	1000	---	0	---	---	---
OPB-01	GTTTCGCTCC	3	777-1417	$OPB-01_{1417}$ $OPB-01_{976}$	66.66	---	$OPB-01_{1417}$ $OPB-01_{976}$	---
OPB-02	TGATCCCTGG	2	759-898	---	---	---	---	---
OPB-03	CATCCCCCTG	2	637-1689	---	---	---	---	---
OPB-04	GGACTGGAGT	2	942-2187	---	---	---	---	---
OPB-05	TGCGCCCTTC	6	539-1653	---	---	$OPB-05_{539}$	---	---
OPB-06	TGCTCTGCCC	6	750-1575	---	0	--	---	---
OPB-07	GGTGACACGG	2	741-1377	$OPB-07_{741}$	50	---	$OPB-07_{741}$	---
OPB-08	GTCCACACGG	8	520-1520	$OPB-08_{1386}$ $OPB-08_{1000}$ $OPB-08_{870}$ $OPB-08_{520}$	50	$OPB-09_{1530}$ $OPB-09_{1164}$	---	$OPB-08_{1386}$ $OPB-08_{1000}$ $OPB-08_{870}$ $OPB-08_{520}$
OPB-09	TGGGGGACTC	0	---	---	0	---	---	---
OPB-10	CTGCTGGGAC	4	956-2156	---	0	---	---	---
Total		**127**	**130-2389**	**22**	**17.32**	**4**	**11**	**11**
ISSR Marker								
UBC-861	$(ACC)_6$	4	---	---	---	---	---	---
UBC-865	$(CCG)_6$	6	445-1000	---	---	---	---	---
UBC-868	$(GAA)_6$	10	539-1889	$UBC-868_{1486}$	10.0	---	---	$UBC-868_{1486}$
UBC-873	$(GACA)_4$	6	505-1541	---	---	---	---	---
UBC-872	$(GATA)_4$	2	1250-3038	---	---	---	---	---
UBC-808	$(AG)_8C$	12	429-2036	---	--	$UBC808_{1058}$ $UBC808_{981}$	---	---
UBC-807	$(AG)_8T$	3	592-924	---	---	---	---	---
		39	**437-3154**	**2**	**2.56**	**2**	**0**	**1**

Table. 5. Details of SCoT markers used for characterization of intraspecific F_1 hybrid showing the inheritance of parent specific markers to the intraspecific F_1 hybrid.

Primer	Sequence (5 ' → 3')	No. of fragments amplified	Range of amplified fragments (bp)	Polymorphic bands	Percentage of polymorphism (%)	No. of Non-parental fragments* in F_1	Parent specific polymorphic bands (bp) in F_1 from	
							CtW2-BL1	*CtB3-SL1*
SCoT -01	CAACAATGGCTACCACCA	5	389-1077	---	---	---	---	---
SCoT -02	CAACAATGGCTACCACCC	7	293-1218	---	---	---	---	---
SCoT-03	CAACAATGGCTACCACCG	12	341-1593	---	---	SCoT-03_{341}	---	---
SCoT -04	CAACAATGGCTACCACCT	4	684-2437	---	---	---	---	---
SCoT -05	CAACAATGGCTACCACGA	3	500-1250	---	---	---	---	---
SCoT -06	CAACAATGGCTACCACGC	8	151-1706	---	---	---	---	---
SCoT -07	CAACAATGGCTACCACGG	6	967-2096	---	---	SCoT-07_{539}	---	---
SCoT-09	CAACAATGGCTACCAGCA	5	394-2260	---	---	---	---	---
SCoT-10	CAACAATGGCTACCAGCC	5	1666-3916	---	---	---	---	---
SCoT-11	AAGCAATGGCTACCACCA	4	509-1255	---	---	---	---	---
SCoT-12	ACGACATGGCGACCAACG	3	366-676	---	---	---	---	---
SCoT-13	ACGACATGGCGACCATCG	2	310-607	---	---	---	---	---
SCoT-14	ACGACATGGCGACCACGC	6	313-1351	---	---	---	---	---
SCoT-15	ACGACATGGCGACCGCGA	2	257-358	---	---	---	---	---
SCoT-16	ACCATGGCTACCACCGAC	5	550-1658	---	---	---	---	---
SCoT-17	ACCATGGCTACCACCGAG	3	590-1546	---	---	---	---	---
SCoT-18	ACCATGGCTACCACCGCC	8	316-1805	---	---	---	---	---
SCoT-19	ACCATGGCTACCACCGGC	14	341-2231	SCoT-19_{583}	7.14	SCoT-19_{548} SCoT-19_{480}	---	583
SCoT-20	ACCATGGCTACCACCGCG	5	500-2210	---	---	---	---	---
SCoT-21	ACGACATGGCGACCCACA	5	231-1023	---	---	---	---	---
SCoT-22	AACCATGGCTACCACCAC	7	269-1132	---	---	---	---	---
SCoT-23	CACCATGGCTACCACCAG	4	275-977	---	---	---	---	---
SCoT-24	CACCATGGCTACCACCAT	6	480-1669	SCoT-24_{562}	16.66	---	---	SCoT-24_{562}
SCoT-25	ACCATGGCTACCACCGGG	8	369-2654	SCoT-25_{1308}	12.5	---	SCoT-25_{1308}	---
SCoT-26	ACCATGGCTACCACCGTC	7	294-963	SCoT-26_{566}	14.28	---	---	SCoT-26_{566}
SCoT-27	ACCATGGCTACCACCGTG	8	527-2386	SCoT-27_{647}	12.5	---	SCoT-27_{647}	---
SCoT-28	CCATGGCTACCACCGCCA	10	474-1552	SCoT-28_{1431} SCoT-28_{981}	20.0	---	---	SCoT-28_{1431} SCoT-28_{981}
SCoT-29	CCATGGCTACCACCGGCC	14	335-2523	---	0	---	---	---
SCoT-30	CCATGGCTACCACCGGCG	8	390-2954	---	12.5	SCoT-30_{438}	---	---
SCoT-31	CCATGGCTACCACCGCCT	11	326-2477	SCoT-31_{774}	9.09	SCoT-31_{326}	SCoT-31_{774}	---
SCoT-32	CCATGGCTACCACCGCAG	9	116-2000	SCoT-32_{1030} SCoT-32_{830}	22.22	---	SCoT-32_{1030} SCoT-32_{830}	---
SCoT-33	CCATGGCTACCACCGCAG	5	210-1000	---	0	---	---	---
SCoT-34	ACCATGGCTACCACCGCA	4	339-1176	---	0	---	---	---
SCoT-35	GCAACAATGGCTACCACC	6	210-2555	SCoT-35 $_{1430}$ SCoT-35_{210}	33.33	---	SCoT-35_{1430} SCoT-35_{210}	---
SCoT-36	GCAACAATGGCTACCACC	5	449-899	SCoT-36_{899}	20.0	---	---	SCoT-36_{899}
	Total	**224**		**13**	**5.80**	**6**	**7**	**6**

CtB3-SL1: 0.104±0.02; *acc. CtW2-BL1:* 0.514±0.019). This variation might be attributed to the genetic recombination favouring conglomeration of suitable alleles, expression of genes producing key enzymes of metabolic pathways and growth environment which probably necessitated the production and accumulation of more taraxerol as reported for different bioactive metabolites in several medicinal species (Amoo and Van Staden 2013).

Mitotic analysis of F_1 hybrid revealed its chromosome count as 2n=16 similar to its parents. Sixteen dis-

tinguished chromosomes were also observed in the mitotic metaphase and they were separated in normal fashion during anaphase. Meiotic analysis revealed formation of eight bivalents at diakinesis and metaphase-I and 8II: 8II separation at anaphase-I, which might be due to homology between the parents. As result the pollen fertility of F_1 hybrid is almost equivalent to its parents. These cytological observations along with morpho-physiological traits could be helpful for the characterization of the F_1 hybrids of *C.* ternatea as reported in many species.

The homologous multigene families control the expression seed protein profile across the species, thus the seed protein marker exhibits monogenic segregation where the presence of polypeptide being completely dominant over absence, and in some cases, co-dominance for molecular weight variants also noticed (Osborn 1988). Mutations or deletions of structural genes coding for these polypeptides or their regulatory loci might lead to lack of expression of the concerned polypeptides (Panigrahi et al. 2007). This kind of variations in seed protein marker profiling led the use this as as reliable markers for verification of hybridity of inter-varietal crosses (Bennet et al. 1991), and inter-specific (Panigrahi et al. 2001, Jisha et al. 2011, Mishra et al. 2012). In the present study SDS-PAGE of seed albumins revealed inheritance of three polymorphic polypeptides (Mw 31.62, 22.38 and 18.81 KDa) specific to pollen parent *C. ternatea acc. CtW2-BL1* in the F_1 hybrid. Since *C. ternatea acc. CtW2-BL1* was used as pollen parent, the appearance of these unique albumin polypeptides in the F_1 hybrid can potentially be used as markers for identification of hybrids involving at least *C. ternatea acc. CtW2-BL1* as pollen parent as reported in *Cajanus cajan* (Panigrahi et al. 2007)

RAPD, SCoT and ISSR marker analysis relies on differential enzymatic amplification of targeted DNA fragments on the basis of primer annealing sequence of the genome. RAPD is being random in nature, this kind of DNA markers were ubiquitously distributed throughout the genome, and capable of detecting a high level of polymorphism. Whereas ISSR is simple sequence repeat specific and SCoT is the specific to the conserved sequence around the initiating codon of the gene. These markers have also been successfully utilized in several crop species for diverse breeding efforts including identification and characterization of the hybrids. In the present study, RAPD, ISSR and SCoT markers showed 17.32, 2.56 and 5.80% polymorphism among the parents. Both ISSR and SCoT analysis showed lower polymorphism (2.56% and 5.80%) in comparison to RAPD markers in the present study. There are some contradictory reports on detection of polymorphism by RAPD and ISSR markers, ISSR markers showed more polymorphism than RAPD markers (Godwin et al. 1997; Lima-Brito et al. 2006; Nagaoka et al. 1997; Zietkiewicz et al. 1994) and vice versa (Muthusamy et al. 2008). This contradiction might be due to the use of different decamer oligonucleotides or SSR motifs as primers, and varied primer-annealing site in the genomes. Again, ISSR and SCoT polymorphism depends on the frequency of SSR motifs and conserved sequence around the initiating codon, respectively (Depeiges et al.1995; Collard and Mackill 2009) which vary within a species or even varieties targeted. Identification of inter and intra-specific hybrids has been carried out in several species using either RAPD or ISSR markers individually, or in combination (Goldmann et al. 2008; Jisha et al.2011; Bianco et al. 2011, Mishra et al. 2012). In this study 22 RAPD, one ISSR and 13 SCoT markers were found to be inherited to the putative F_1 hybrid, and among them 11 RAPD and six SCoT markers, unique to pollen parent (*C. ternatea*, *acc. CtW2-BL1*), were very vivid in its appearance for the identification of F_1 hybrid. In the present study, several non-parental fragments have also been amplified in the F_1 hybrid, and this might be due to either DNA recombination followed by minor genomic reorganization during the hybridization (Huchett et al. 1995), or loss of priming sites due to chromosomal crossing over during meiosis (Smith et al.1996). As the objectives is to identify the hybrids and to confirm the hybrid nature of putative seedlings at the juvenile stage, screening of the putative F_1 hybrids using pollen parent-specific RAPD, ISSR and SCoT markers contribute economic significance to this medicinal plant. The findings from the present study, it has been assorted that use of seed protein profiling and DNA marker analysis complements the characterization of intra-specific F_1 hybrid along with morpho-physiological traits and cytogenetic analyses more precisely. Further, these inherited seed albumin and DNA markers could also be used for further studies in gene mapping, marker-assisted breeding involving intra-specific hybridization in *C. ternatea* aiming at enhanced metabolite content of therapeutic importance.

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