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Distinctness, uniformity and stability testing in Moringa- An overview

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Abstract
The Govt. of India enacted "The Protection of Plant Varieties and Farmers' Rights (PPV&FR) Act, 2001" adopting sui generis system. Registration and protection can be granted to a variety only if it conforms to the criteria of Distinctness, Uniformity and stability (DUS). It means that the new variety has to be DUS in its characteristics. This requires the examination of the variety if it conforms to the standards of DUS test. The examination of a variety for DUS generates a description of the variety, using its relevant characteristics. Very meager work is done in medicinal plants in general and in *Moringa* species for DUS characterisation. Therefore a DUS characterization procedure has been discussed for germplasm characterisation and evaluation. Descriptors for evaluation consist of traits of crop husbandry values and response to abiotic and biotic stresses.

Keywords: Characterization, Drumstick, DUS, Markers, Plant Variety Protection

Introduction
Moringa oleifera is grown throughout India, usually as a multipurpose medicinal plant, and is cultivated widely for its immature fruit as well as leafy vegetable, stems, roots, pods and seeds. All parts of *Moringa oleifera* are economically viable as food and nutrition, animal fodder, natural coagulants, forestry products and fertilizer (Afolabi *et al*., 2013; Ahmad *et al*., 2014) [3, 4]. In addition, the leaves and the seeds are nutritionally rich containing high concentrations of crude protein, calcium, iron, potassium, manganese, essential vitamins (thiamine, riboflavin, niacin, ascorbic acid), anti-oxidants and anti-inflammatory compounds (Anwar *et al*., 2007; Adisakwattana and Chanathong, 2011) [6, 2]. The medicinal uses, safety and efficacy of *M. oleifera* have been widely reported by several authors (Anwar *et al*., 2007; Hussain *et al*., 2014; Stohs and Hartman, 2015) [6, 15, 28]. The seed oil as raw material for production of biodiesel is gaining attention globally as possible replacement for petro diesel fuel in unmodified engines (Fernandes *et al*., 2015) [11]. However, *M. oleifera*, as a vegetable, is considered neglected and underutilize species (NUS) in some parts of the world (Padulosi *et al*., 2013a; Rudebjer *et al*., 2013) [18, 22]. The negligence may be probably due to little or no research attention from agronomic researchers and policy makers, loss of local knowledge and lack of established varieties (Padulosi *et al*., 2013) [18]. Usually, NUS are not traded as commodities; however, the economic importance of *M. oleifera* has elicited utilization in different parts of India.

Understanding its nutritional and medicinal importance, many SAUs, institutions have initiated crop improvement program from 1980s and as a result of the efforts of many scientists, numerous improved varieties of drumstick are developed and released for farmer's cultivation particularly in western and southern parts of India. The effective system for protection of plant varieties developed primarily based on the broad principles of distinctiveness, uniformity and stability. A variety has to pass through DUS test compulsorily for registration under PPV&FRA. The descriptors and guidelines to conduct DUS test are developed by UPOV and Biodiversity international or NBPGR for major agricultural and horticultural crops. It is an effective system of Plant Variety Protection (PVP) will not only be a safeguard against unlawful commercial exploitation of the new varieties but also stimulate the development of new varieties. In order to provide for the establishment of an effective system for the protection of plant varieties, the rights of farmers and plant breeders and to

encourage the development of new varieties of plants it has been considered necessary to recognize and to protect the rights of the farmers in respect of their contributions made at any time in conserving, improving and making available plant genetic resources for the development of new plant varieties.

Plant variety protection

The Indian Patents Act of 1970 does not permit patenting of plants or varieties. Patents on plants, considered a strong form of protection, are available in advanced countries like USA, Japan under certain conditions. But in developing countries, patents are replaced by PVP (Plant Variety Protection) in recognition of the fact that 'variety development 'involves improvement of already existing ones and not de novo creation. Accordingly, India had enacted its own PVP law, "Protection of Plant Varieties and Farmers' Rights Act" in 2001, with provisions for protecting both breeders and farmers rights. The plant variety protection is directly related to innovations in agriculture field, it must also be understood in broader context that it includes conservation of biological resources (Phillippe, C. and Radhika, K., 2003) [19]. This act provides protection of new varieties including extant and farmer's varieties. The grant of plant breeders rights (PBR) entitle the breeder (or his successor, agent, licensee) to exclude others from producing, selling, marketing, distribution, export or import of propagating material of protected varieties for a period of 15 years for annuals and 18 years for vine and trees. The act also permits a breeder to use a protected variety for research purpose. The act allows the farmer to save, sow, resow, exchange, share or sell farm produce including seed of a protected variety.

DUS Testing

Registration and protection can be granted to a variety only if it conforms to the criteria of Distinctness, Uniformity and stability. It means that the new variety has to be Distinct Uniform-Stable (DUS) in its characteristics. This requires the examination of the variety if it conforms to the standards of DUS test. The examination of a variety for DUS generates a description of the variety, using its relevant characteristics. This examination of a variety is either conducted by the Plant Variety Protection Authority ('Official testing') or by the breeder seeking protection ('Breeder testing'). In some countries (Japan, New Zealand), both government or official testing and breeder testing are done. Official testing is common in European countries. Under breeder testing (as in USA, Australia), the applicant has to conduct the tests and demonstrate to the PVP examiner that his new variety meets the criteria of distinctness, uniformity and stability.

Distinct-Uniform-Stable (DUS)

Distinct means a variety should be clearly distinguishable by one or more essential characteristics from any other existing variety. Diversity in plant architecture, growth and branching habits as well as yield and yield characteristics observed in the study are desirable. That is, diversity provides the breeder with genetic material for selection and improvement of the plant (Brothers and Kelly, 1993; Acquaah *et al.,* 1992) [7, 1]. For instance, selection for high yield among these accessions could focus on either BNR8 or BNR6 varieties of drumstick towards progressive increase in yield over time. (Amoatey *et al*., 2012) [5]. The knowledge of genetic diversity of tree crop is very important for rational planning of conventional, modern breeding and improvement programme for the purpose of improving the yield and its quality of produce (Rafii *et al.,* 2012) [20].

The variety is deemed Uniform if it is sufficiently uniform in its relevant characteristics, subject to variation that may be expected from the particular features of its population. The basis of assessment is normally the number of off-types in the variety, judged on the basis of a population standard and an acceptable probability fixed in the corresponding species. To identify off-types in a population, generally visual observation on characteristics may suffice. However, in a few cases/ crops, it may be necessary to make measurements of each plant to apply statistics to decide or not whether a plant is an off-type. In most of the crops, acceptance probability of 95% has been suggested. For vegetatively propagated and self-pollinated varieties, the following standard has been suggested:

Sample size	Off-types (permissible)
<5	0
6-35	1
36-82	2
83-137	3

Generally, cross-pollinated varieties exhibit wider variation within the variety. Relative tolerance limits can be found by comparing with comparable varieties. The standard deviation or variance may be used as the criteria for comparison. Recently, UPOV has proposed a statistical method called 'Combined Over Year Uniformity (COYU)' that takes into account variations between years for dealing uniformity in measured (quantitative) characters.

The variety is said to be Stable if its relevant characteristics remain unchanged after repeated propagation. Though it is not usually possible to assess stability with in a period of 2 or 3 years, the variety can be considered stable if is shown to be uniform. Environmental stability estimates were determined using the linear regression model procedure based on an environmental index (Eberhart and Russell, 1966) [9]. In this procedure, the response of a genotype to a number of environments was compared to the other genotypes in the study using three values such as genotype mean over all environments (X), regression coefficient *(b* value) and deviation mean square which is the deviation from the linear regression (S^2di) line over all environments. The importance of stability estimation has more values in perennial crops such as drumstick (*Moringa oleifera* Lam.), as it has occupied the area for many years. Stability analysis was assessed among 14 drumstick genotypes, which revealed that PKM-2, MO-1 and PKM-1 were found stable for number of fruits per plant and yield due to bi value (regression coefficient) around unity and non-significant S^2di (deviation mean square) value. Hence, PKM-2 and MO-1 were found to fit for favourable environment and PKM-1 for unfavourable environment for commercial cultivation for semi-arid region of India (Raja S., *et al*., 2013) [21].

Characterization

The requirement of distinctness, uniformity and stability are assessed on the basis of characteristics. The characteristics are a feature of whole plant or part of plant. Such characteristics may be morphological, biochemical, molecular or any other nature. The table of characteristics chosen by experts forms the main part of test guidelines and of DUS testing. In Genetic resources, the term 'characteristic' is known as descriptors (with descriptor states) and describing a plant based on such descriptor is known as 'characterization'.

a) Morphological characterization: This is based on botanical or morphological descriptors or characters of the plant or plant part. Before a variety is registered as a cultivar and/or granted Plant Breeder's Rights, its distinctness, uniformity and stability (DUS) is tested using morphological characters (descriptor). Fourteen quantitative characters of 300 genotypes of drumstick trees belonging to 12 populations located at Tamil Nadu (southern part of India) and Himachal Pradesh (Northern part of India) were recorded and mean values based on five trees observation, revealed that the tree height (4.24 m), Trunk girth (1.36 m), tree spread (E–W) (3.65 m) and tree spread (N–S) (3.25 m) showed good morphological variation. Fruit characters such as fruit length (7.05 cm), fruit girth (8.52 cm), fruit weight (110.0 g), number of fruits per tree (79.50), number of seeds per fruit (20.17) and oil content (28.29) were also varied between the population (Ganesan S. K. *et al.,* 2014) [12].

At 12 months after planting (MAP), mean plant height ranged between 4.7 m and 7.4 m while mean girth ranged between 5.2 cm and 9.7 cm. For both parameters, the lowest values were recorded by drumstick genotypes BNR15 while BNR10 and BNR6 had the highest height and girth, respectively. Mean canopy radius ranged from 2.32 m to 3.96 m. There were significant differences (P_0.05) among moringa accessions for mean plant height, girth and canopy radius. The overall mean number of days from transplanting to 50% flowering was 152.9 days, whilst overall mean number of days to 50% fruiting was 257.6. Variations in flowering and fruiting frequency among moringa accessions place them in three categories: (i) those that flowered and fruited twice within the year with two peaks, (ii) those that flowered twice within the year but with only one fruiting peak and (iii) those that flowered and fruited only once yearly.

Types of characteristics

1. Qualitative characteristics: Truely qualitative characteristics show discrete discontinuous states and are stable, heritable and uniformly expressed in all environments (Ex. Shape, Flower colour, etc). According to Jacob O. P. *et al.,* 2016, drumstick flowers are papilionoid with 5 stamens and described as monosymmetric. Amongst all the accessions studied, the flower colour was generally white with purple pigmentation or creamy white without pigmentation. Only 4 accessions were observed to possess white with purple pigmentation (enN053, goN068, knN078 and knN077). The fresh pod color was pale green in 25 accessions while it was green in 15 accessions. At maturity, all pods were brown in colour except few that were scored to be golden brown. Fruit shape was straight in all the accessions except in accessions abN057, anN049, anN051 and niN018 that were scored to be curved shaped.

2. Pseudo-qualitative characteristics: Here, the range of expression is at least partly continuous varying in more than one dimension. In some cases, intermediate states of expression such as 'weakly expressed' are included between 'absent' and 'strongly expressed'. (Ex. Pubescence, Pigmentation, etc.).

Qualitative characteristics are assessed visually while quantitative characteristics are usually measured. The following types of assessments are recommended:

- VG: Visual assessment by a single observation of a group of plants or parts of plant.
- VS: Visual assessment by observation of individual plants or parts or plants.
- MG: Measurement by a single observation of a group of plants or parts of plant.
- MS: Measurement of a number of individual plants or parts or plant.

Example: Characteristics of Bael tree

S. No.	Bael tree	State	Note	Example variety	Stage of observation	Type of assessment	Reference
1	Leaf colour	Light green	3	Pant Shivani, Pant Urvashi	a	VG	A.K. Singh *et al*., 2011 [25]
		Green	5	CISHB-1, CISHB-2, NB-16, NB-17			
		Dark green	7	NB-5, Pant Aparna, NB-16			
2	Fruit shape	Globose	1	Goma Yashi, Pant Shivani, Pant Urvashi	c	VS	
		Ovate	3	CISHB-1, NB-9			
		Elliptical	5	CISHB-2, NB-7, Pant Urvashi			
		Round	9	Pant Aparna, NB-16, Pant Sujata			
3	Leaf size	Small	3	Pant Urvashi, NB-9, NB-17, NB-5	a	MS	
		Medium	5	Pant Sujata, Goma Yashi, Pant Aparna			
		Large	7	NB-7, Pant Shivani			
4	Inter nodal distance	Low (<3cm)	3	NB-5	a	MG	
		Medium 3-3.5 cm	5	Pant Sujata, Pant Urvashi, NB-17, Goma Yashi, NB-16			
		High > 3.50 cm	7	NB-9, NB-7, CISHB-1,CISHB-2, Pant Aparna			

b) Grouping characteristics: can be universally used, either individually or collectively, for grouping the similar varieties. These characteristics are considered to be most reliable in distinguishing or discriminating varieties.

Principal components analysis (PCA) confirmed the results of the cluster analysis showing a high genetic relationship among the accessions. High genetic diversity implies that some of the accessions with superior characteristics may be chosen as parents in future breeding programmes (Gitonga *et al.* 2008; Varalakshmi, 2007) [13, 29].

c) Biochemical characterization: Isozyme based descriptors have been widely used for identification of crop varieties because of their reliability (Smith & Smith 1992, Cooke, 1995) [27, 8]. UPOV has also included electrophoresis of isoenzymes in maize, soybean, sunflower and of seed proteins in barley, wheat as additional characters for establishing distinctness of varieties. Selection of an appropriate electrophoresis technique provides a potential tool for variety identification, DUS test or grouping of varieties.

Moringa seed kernels contain a significant amount of oil that is commercially known as Ben oil, which has been used for

illumination and is considered to be particularly suitable as a lubricant for fine machinery. Ibrahim *et al*., 1974, reported that the oil content and its properties are varied over a wide range, mainly depending on the species and environmental conditions. The hexane-extracted oil content of *Moringa oleifera* seeds ranged from 38.00 to 42.00%. Protein, fiber, and ash contents were found to be 26.50-32.00, 5.80-9.29, and, 5.60-7.50%, respectively (Farooq Anwar and M. I. Bhanger, 2003) [10].

d) Molecular characterization: Biotechnology has widened the possibilities for applying such technologies to the problem of characterization, varietal identification and protection (Smith, 1995). Molecular markers are used for development of saturated genetic maps; DNA fingerprinting; phylogenetic and evolutionary studies; heterotic breeding; gene tagging and marker assisted selection (MAS). The two commonly adopted approaches in the use of molecular markers are essentially either probe based such as RFLP (Restriction Fragment Length Polymorphism), or amplification based like RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), STMS (Sequence Tagged Microsatellites), *etc*. They are reliable, fast and cost effective to discriminate an EDV (essentially derived variety). Data from these methods may help resolve disputes on identity of germplasm or hybrids (Santhy *et al.*, 2003) [23].
The morphological (qualitative and quantitative) and SSR makers had been used for characterisation of 300 genotypes of drumstick of India; which belongs to 12 distinct populations. The study with morphological (qualitative and quantitative) and SSR makers showed that large diversity exists in Indian germplasm. At molecular level, 19 SSR primers that produced clear and reproducible bands were selected for diversity study. A total of 35 bands were amplified of which 29 (82.86%) were polymorphic with an average of 1.84 bands per primer (Ganesan S.K. *et al.*, 2014) [12].
Thirty four genotypes of *Moringa oleifera* found in different regions of Tamil Nadu state in India using morphological and molecular markers. 14 markers were used for screening of 34 genotypes of moringa for DNA profiling using SSR primers revealed polymorphism among the 34 genotypes assessed. The characterization resulted in the identification of MO-56 as the superior parent followed by MO-6 and MO-1 for involving in hybridization programme to improve the oil yield. (Natarajan S. and Aslin Joshi, 2015) [17].

Types of scales of data
DUS depends on the level of scales of data, which are recorded for the characteristics. Scale may be quantitative or qualitative.

Quantitative scaled data
The data that are recorded by measuring or counting is said to be quantitative scaled data. This data can have continuous or discrete distribution. Continuous data results from measurement. Discrete quantitative data result from counting.

Example -Continuous: Plant length in cm. – measurement - Discrete: Number of stamens (1, 2, 3, 4 and so on).
In discrete quantitative data there are no real values between two neighboring units but is allowed to compute an average, which is in between these units. The 14 moringa accessions were also scored based upon expression of phenotypic and morphological traits (using a scale of 1-5 where 1 = best and 5 = worst) (Amoatey *et al*., 2012) [5].

Qualitative scaled data
Qualitatively scaled data are data, which can be arranged, in qualitatively different categories. Usually they are based on visual assessment. Qualitatively scaled data is further divided into ordinal (qualitative underlying quantitative variables) and normal scales.

i) Ordinal scale
Qualitative data (qualitative underlying quantitative variables) in which discrete can be arranged in an ascending or descending order
Example: intensity of anthocyanin

ii) Nominal scale
Nominal scale qualitative data are data without any logical order of the discrete categories.

Example: Sex of plant: (1) dioecious female, (2) dioecious male, (3) Monoecious unisexual, (4) monoecious Hermaphrodite; Leaf blade: non-variegated, variegated.
A nominal scale consists of numbers which correspond to the state of expression of the characteristics in the test guidelines as notes. Characteristics with only two categories (alternative characteristics) are a special form of nominal scales.

Conclusion
Registration and protection can be granted to a variety only if it conforms to the criteria of Distinctness, Uniformity and stability. It means that the new variety has to be Distinct Uniform-Stable (DUS) in its characteristics. This requires the examination of the variety if it conforms to the standards of DUS test. The examination of a variety for DUS generates a description of the variety, using its relevant characteristics. This examination of a variety is conducted by the PPV&FRA. Thus, an effective system of Plant Variety Protection (PVP) will not only be a safeguard against unlawful commercial exploitation of the new varieties but also stimulate the development of new varieties.

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