

Characterization of *Ocimum tenuiflorum* (Linn.) Morpho-types Using RAPD Markers

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Abstract

Ocimum tenuiflorum Linn. (Lamiaceae) is an aromatic plant with a lot of potential medicinally, industrially and domestically. There are different morpho-types of *O. tenuiflorum*, with different shades of purple pigment on their leaves. Very few studies have been done to study the genetic variation among *O. tenuiflorum* morpho-types. Thus, in the present study, four *O. tenuiflorum* morpho-types that varied in pigmentation were characterised using RAPD marker as a molecular tool. Eight different primers collectively amplified 64 bands in the four morpho-types analysed. The RAPD technique revealed that the varied amount of purple pigment in *O. tenuiflorum* morpho-types is not due to environmental factor alone. The UPGMA clustering algorithm based on RAPD data grouped the four morpho-types into two major groups, with *O. tenuiflorum* (T2) with very light purple leaves and stem morpho-type, more related to *O. tenuiflorum* (T3) that have deep purple leaves and stem. The result was able to show the phylogenetic relationships within the species. The low level of genetic diversity observed in *O. tenuiflorum* can be attributed to the mode of reproduction and the reproductive biology of the species, due to the fact that they are likely to be natural hybrids produced through cross pollination.

Keywords: morpho types; *Ocimum tenuiflorum*; primers; RAPD

Introduction

Ocimum tenuiflorum Linn. (Lamiaceae) is an aromatic plant with about 150 variants. It is one of the most famous perennial herbs which are native to India and widespread as a cultivated plant and an escaped weed, covering the entire Indian sub-continent (Kirtikar and Basu, 1984). It is largely distributed in Asia, Australia and West Africa and also in some Arabian countries, mainly in drier sandy areas (Pistrick, 2001). *O. tenuiflorum* is also known as *O. sanctum*. It is called Indian's holy basil in English, 'Kala-Tulsi' among the Hindi-speaking people of India and 'Efinrin wewe' among the Yoruba speaking people of South-western Nigeria (Mohan *et al.*, 2011).

It is an erect, tall, sub-shrub plant, with hairy sub-quadrangular branches. Leaves are simple, ranging from shades of green to purple. The leaves can be ovate, elliptic-oblong, obtuse or acute in shape. The aerial parts possess glandular hairs on stalked and sessile glands which secrete volatile oils. The inflorescence has purplish-white, hermaphroditic and zygomorphic flowers arranged in

elongate racemes in close whorls. They produce numerous tiny seeds which are dark brownish and globose-subglobose in shape with shiny seed coat. *O. tenuiflorum* is readily distinguished from the other species in the genus *Ocimum* by their characteristically spreading pedicels and the internally glabrous calyces (Malav, 2015).

The variants of *O. tenuiflorum* with different coloured flowers, with combinations of purple or green calyces and purple or white corolla are available. Four main morpho-types have been identified in South-west Nigeria. Morphologically indistinguishable types represented from diverse ecological regions had variable chemical constituents (Ali and Ali, 2012). A wide variability within different traits was observed in accessions of population from diverse phyto-geographical regions varying in climate, habit and morphology (Malav, 2015). The variability observed in *Ocimum sanctum* genotypes on the basis of morpho-molecular investigations indicated the genetic diversity among natural populations/genotypes found in the district Poonch, Azad Kashmir (Ahmad and Khaliq, 2002). Variability based on pigmentation exists also within the morpho-types of *O. tenuiflorum*. Despite the potential of pigmented traits as genetic markers, no concrete genetic studies have been done on these traits on *O. tenuiflorum* in Nigeria.

Materials and Methods

Plant material

Fresh leaves of four morpho-types of *O. tenuiflorum* i.e., *O. tenuiflorum* (green leaves and stem) which was tagged (T₁), *O. tenuiflorum* (very light purple leaves and stem) (T₂), *O. tenuiflorum* (deep purple leaves and stem) (T₃) and *O. tenuiflorum* (light purple leaves and stem) (T₄) were collected at the experimental garden of the Department of Botany, Obafemi Awolowo University, Ile Ife, Nigeria.

DNA extraction

Total genomic DNA was extracted from fresh leaf tissues by the method of Doyle and Doyle (1990) with slight modification as explained below.

The samples were prepared by putting approximately 100 mg of fresh leaf tissues into an extraction tube. Two steel balls were added each into the tube to enable grinding. The tissues were ground into fine powder by using Genogrinder-2000. Thereafter, 450 µl of pre-heated plant extraction buffer was added. Incubation of the tubes was done at 65 °C for 20 min after which the tubes were mixed by occasionally inverting them to homogenize the sample. The tubes were removed and allowed to cool for 2 min, 200 µl of ice-cold 5M potassium acetate was then added. The tube was incubated on ice for 20 min to precipitate protein and centrifuged at 3,500 rpm for 10 min and the supernatant was then transferred into freshly labeled tubes. Then, 2/3 volume of ice-cold isopropanol was added into the tubes, mixed gently and incubated at -80 °C for 15 min to precipitate the DNA. It was then centrifuged at 3,500 rpm for 15 min. The supernatant was decanted until the last drop. About 400 µl of 70% ethanol was added to wash the DNA pellet, then centrifuged at 3,500 rpm for 15 min. The supernatant was decanted until the last drop and the pellet was air dried (until ethanol smell disappears). 60 µl of ultra-pure water or (low salt TE) was added to re-suspend the DNA. 2 µl of RNase was added and incubated at 37 °C for 30-40 min. In order to check the DNA quality and removal of RNA, 0.8% agarose gel was prepared by boiling 0.8 g of agarose in 100 ml of 1X TBE, and allowed to cool at 60 °C; then, 5 µl ethidium bromide was added and gently swiped to mix, the solution was poured on the gel tray before it polymerizes. 3 µl of DNA and 3 µl of loading dye were mixed together and 6 µl of the mixture was loaded on to the 0.8% agarose gel. The gel was run at 80 V for 60 min and the gel picture was saved. When the RNA was completely removed it was then proceed to the Nanodrop. DNA concentration was quantified using DNA-50 option of the Nano drop spectrophotometer.

PCR condition

PCR reaction was performed using eight commercial primers (Table 1). Optimal conditions for RAPD analysis were determined by the highest number of fragments and accurate bands. PCR was performed in a 25 µl volume of reaction mixture (Williams *et al.*, 1990) containing 1X *Taq* polymerase buffer (with 25 mM MgCl₂), 0.6 units of *Taq* DNA polymerase, 5 mM dNTPs, 10 mM of ransom decamer primer (Finnzymes) and 15 mg of total genomic DNA. Amplifications were carried out using a DNA

thermo cycler (Bio-Rad Laboratories, USA) with the following parameters: 1 cycle of 4 min at 94 °C followed by 45 cycles of 1 min at 94 °C, 1 min at 37 °C and 2 min at 72 °C. The last extension cycle was programmed at 72 °C for 7 min. The PCR products were resolved on a 1.5% agarose gel containing 0.4 mg/L ethidium bromide and the photograph was taken and documented.

Statistical analysis

The position of the amplified bands was compared for each morpho-type and primer combination. The non-reproducible and ambiguous bands were eliminated. The photograph was converted into a binary matrix using "1" and "0" characters. The bands were scored "1" for presence and "0" for absence. Further analysis was done by calculating the Pairwise Nei Genetic Distance and Pairwise Nei Genetic Identity using Genalex 6.502 software and MEGA 7 software. The dendrogram was constructed using the Unweighted Pair Group Method using Arithmetic means (UPGMA) (Sokal and Sneth, 1973).

Results

In the present study, four *O. tenuiflorum* morpho-types were surveyed with RAPD marker system. Eight arbitrary primers were used and they were all polymorphic. The 8 primers used gave a total of 64 bands across the 4 morpho-types. The number of total amplicons varied from 13 for *O. tenuiflorum* (T₁) to 20 for *O. tenuiflorum* (T₃). *O. tenuiflorum* (T₁) morpho-type, *O. tenuiflorum* (T₂) morpho-type and *O. tenuiflorum* (T₄) morpho-type had the same number of private bands (3) (bands that are unique to each morpho-type and cannot be found in any other morpho-type), whereas *O. tenuiflorum* (T₃) morpho-type had one more private band (4) than the other morpho-types studied. *O. tenuiflorum* (T₃) morpho-type had the highest number of common bands (16), followed by *O. tenuiflorum* (T₄) morpho-type (14), then by *O. tenuiflorum* (T₂) morpho-type (11), while *O. tenuiflorum* (T₁) morpho-type produced the lowest number of common bands (10).

Discussion

The result of Pairwise Nei Genetic Distance analysis is presented in Table 2. A dendrogram was constructed based on pairwise morpho-type matrix using Unweighted Pair Group Method with Arithmetic averages (UPGMA) (Sokal and Sneth, 1973). The tree (dendrogram) with the sum of branch length of 1.42240475 is shown in Fig. 1. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of Nei Genetic Distances used to infer the phylogenetic tree.

The UPGMA clustering algorithm based on RAPD data grouped the four morpho-types into two major groups (Fig. 1), with *O. tenuiflorum* (T₁) morpho-type and *O. tenuiflorum* (T₄) morpho-type in one group and *O. tenuiflorum* (T₂) morpho-type and *O. tenuiflorum* (T₃) morpho-type in the other group. Based on the Pairwise Nei Genetic Distance analysis of the RAPD data, it can be stated that *O. tenuiflorum* (T₁) morpho-type is more related to *O.*

Table 1. List of primers used and their sequence

Primers	Sequence
OPB03	5' - CAT CCC CCT G - 3'
OPB08	5' - GTG CAC ACG G - 3'
OPB13	5' - TTC CCC CGC T - 3'
OPB14	5' - TCC GCT CTG G - 3'
OPH05	5' - AGT CGT CCC C - 3'
OPT01	5' - GGG CCA CTC A - 3'
OPT17	5' - CCA ACG TCG T - 3'
OPT20	5' - GAC CAA TGC C - 3'

Table 2. Pairwise *O. tenuiflorum* morpho-types matrix of Nei genetic distance

	T ₁	T ₂	T ₃	T ₄
T ₁	0.000			
T ₂	0.624	0.000		
T ₃	1.030	0.767	0.000	
T ₄	0.442	0.934	0.963	0.000

(T₁) *O. tenuiflorum* with green leaves and stem, (T₂) *O. tenuiflorum* with very light purple leaves and stem, (T₃) *O. tenuiflorum* with deep purple leaves and stem), (T₄) *O. tenuiflorum* with light purple leaves and stem

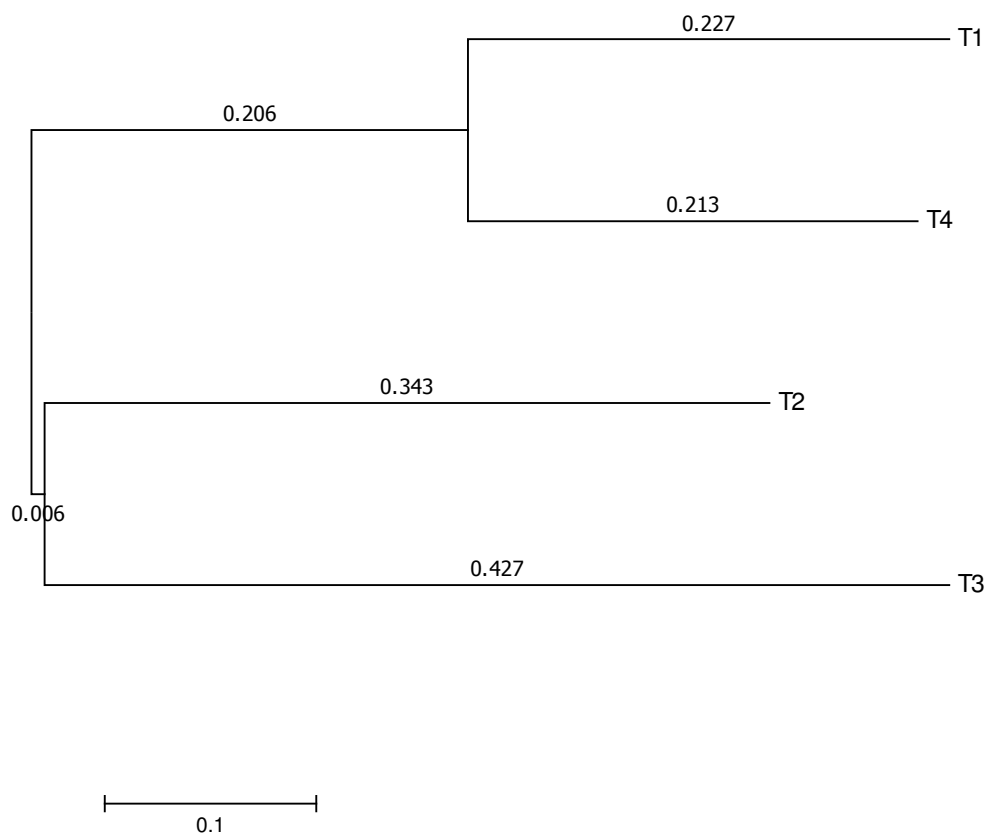


Fig. 1. The UPGMA clustering algorithm based on RAPD data of *O. tenuiflorum* morpho-types; (T₁) *O. tenuiflorum* with green leaves and stem, (T₂) *O. tenuiflorum* with very light purple leaves and stem, (T₃) *O. tenuiflorum* with deep purple leaves and stem), (T₄) *O. tenuiflorum* with light purple leaves and stem

tenuiflorum (T₄) morpho-type than *O. tenuiflorum* (T₂) morpho-type and *O. tenuiflorum* (T₃) morpho-type.

Also, *O. tenuiflorum* (T₂) morpho-type is more related to *O. tenuiflorum* (T₃) morpho-type than *O. tenuiflorum* (T₁) morpho-type and *O. tenuiflorum* (T₄) morpho-type (Fig. 1).

The results from the present study revealed that RAPD markers successfully differentiated the four morpho-types of *O. tenuiflorum*. The number of polymorphic bands indicated genetic variation within *O. tenuiflorum* morpho-types, which showed that the morphological variations were genetic. In the hereby study, the size of amplified fragments varied within the different primers used; this result is in correlation with the study of Ibrahim *et al.* (2013) who observed genetic dissimilarity among the morpho-types of *Ocimum* studied using morphological traits and RAPD markers. Savat *et al.* (2016) also reported substantial genetic variation within species and between species. They concluded that the genetic base of *Ocimum* genus is quite broad. In the current study, the number of genetic loci detected with RAPD markers are much higher than those detected with morphological and biochemical markers. Jakar *et al.* (2015) reported the usefulness of molecular study in differentiating individuals that are closely related.

The low level of genetic diversity observed in *O. tenuiflorum* can be attributed to the mode of reproduction and the reproductive biology of the species, due to the fact that they are likely to be natural hybrids produced through cross pollination (Oziegbe *et al.*, 2016). Individuals that undergo cross pollination have higher gene diversity compared with self-pollinating species (Alvarez *et al.*, 2001).

Conclusions

The identification of primers that can apparently generate species specific profiles is significant for further phylogenetic studies in *Ocimum*. RAPD techniques indicated that it is useful in estimation of polymorphism and phylogenetic relationships among *O. tenuiflorum* morpho-types. The polymorphism data generated can be used for further breeding and characterisation of the species.

Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

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