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Characterisation of Fourteen Accessions of *Trichosanthes cucumerina* from Nigeria Using Internal Transcribed Spacer

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ABSTRACT

Snake gourd botanically known as *Trichosantes cucumerina* locally called tomato agwo. *T.cucumerina* is considered a neglected and underutilised crop. Detailed characterisation of this crop is not known in Nigeria and other African countries. This study was done to access fourteen accessions of *T. cucumerina* from Nigeria using Internal Transcribed Spacer (ITS). The fourteen accessions from different ecological regions were sequenced with ITS1 and ITS4 by direct PCR products. The results obtained were at minimum (570) and maximum (580) base pairs. The query coverage ranging from 99.4 % to 100 % affirms positive amplification and sequencing of snake gourd ecotypes using ITS1 and ITS4. The phylogenetic tree of the characterization group *T. cucumerina was divided* into three clusters. The phylogenetic tree of the characterization grouped *T.cucumerina* into three clusters. The genetic distances between the samples revealed that Ukwa, NACGRAB, Ikwuano, Oshogbo, Ikom, and Rumibekwe were closely related. Benin, Nasarawa, Oye-Ekiti, Ilorin, and NHST-0583 shared no genetic relationship as revealed by their genetic distances. The genetic distance ranged from 0.217 - 2.010. *T. cucumerina* landraces studied in Nigeria thirteen were *T. cucumerina* var *anguina*. The NHST -0583 from the seed center was the only *T.cucumerina* var *cucumerina* in Nigeria. This was blasted on the National Center for Biotechnology Information (NCBI) website. The sequenced blast in the NCBI data website was to be similar to accession numbers GU059528.1 and GQ240883.1 respectively. These have given scientists critical knowledge regarding *T. cucumerina* molecular breeding in Nigeria.

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Introduction

In Nigeria, indigenous people traditionally use a wide range of plants as food and medicine. The *Cucurbitaceae* family is genetically diverse; it contains about 130 genera and approximately 900 species, mainly distributed in tropical and sub-tropical regions (Onyenike *et al.*, 2020). The cucurbits family is one of the economically important groups of plants, including most species providing humans with edible products and beneficial fibers (Bisogonin, 2002). Also in the genus, important economically cultivated crops can be found, such as Cucurbits, Citrillus, Lagenaria, Momordica, and Trichosanthes (Whitaker and Davies, 1962; Dhiman *et al.*, 2012; Ajuru and Nmom, 2017). Cucurbit fruits are very essential for human health. Snake Tomato (*Trichosanthes cucumerina*), also known as viper gourd or long tomato, is a well-known plant with fruit mainly consumed as a vegetable (Liyanage *et al.*, 2016). Morphological studies have been useful in getting information on

genetic studies but this method has limitations in measuring phenotypic characters which are affected by environmental and climatic factors. Genetic diversity found in species showed a valuable genetic resource for breeding and genetic studies (Roberts *et al*., 2018).

DNA barcoding is a tool for species identification. It can be done by amplifying and sequencing a specific region of DNA, which will lead to the creation of a global database of living organisms (Hebert *et al.*, 2003). It has become a useful tool for biodiversity investigation, monitoring molecular phylogeny and evolution (Yong *et al*., 2017). Internal transcribed spacer (ITS) sequence of nuclear ribose deoxyribonucleic acid (nrDNA) has been widely used in resolving phylogenetics among closely related species of angiosperms (Chattopadhyay *et al*., 2017). The ITS region of ribosomal DNA (rDNA) is a component of transcriptional units that code for 18 S, 5.8 S, and 2.8 S, separated by ITS-1 and ITS-2, respectively. It is surrounded by an intergenic spacer (IGS). In most angiosperms, ITS exist in several hundred copies and are located in one or several loci and distributed in the chromosomes. There are three parts to it: ITS1, ITS2, and 5.8S. ITS1 has a higher discrimination capacity, while 5.8S is the most conserved gene (Hollingsworth *et al*., 2016). Qun *et al.* (2021) reported a new species of *Trichosanthes sunhangii* from China using three DNA barcodes which are ITS, matK and rpl20-rpls2 and observed that two accessions of *T.sunhangii* were classified together with Foliobracteola. Cahyaningsih *et al*. (2022) reported the identification of 61 medicinal plant species from 30 families and a pair of ITS2, matK, rbcl and trnl were used for DNA barcoding studies. The result yielded 212 barcoding sequences and identified new ones for the studied medicinal plant species. The study recommended matK for Indonesia medicinal plant identification and ITS2 and rbcl as alternative complementary regions. Ningrum *et al*. (2020) reported the genetic variability of *Begonia longifolia* Blume from Indonesia using nuclear DNA internal transcribed spacer 2 (ITS2) sequence data and observed that the fourteen (14) specimens were grouped into two groups. Hashim *et al.* (2021) studied the phylogenetic relationship and DNA barcoding of nine endangered medicinal plants in the Saint Katherine protectorate. The barcodes used were rbcl, ITS, ycfl and Scot. The barcodes were able to identify the genetic relationship between the nine species. Rbcl identified eight out of the nine to the species level while ITS identified all nine species to the genus level. Huan *et al*. (2018) reported on how to create the DNA barcodes for *Magnolia chevalieri* by using *MatK*, *rbcl*, *trnH-psbA*, *ITS*2, and *ycflb*. Observations made were matK and rbcl similarity was very high trnH-psbA and ycflb were also highly similar. The authors recommended ITS2 and trnH-psbA markers as DNA barcodes for the species.

Nevertheless, none of the studies on molecular bioinformatics with barcodes have identified Nigerian landraces of snake gourds. Therefore, the current study's goal is to use ITS to characterize fourteen *T. cucumerina* accessions in Nigeria.

Materials and Methods

The study focused on the genetic diversity of *T. cucumerina* in Nigeria using seeds collected from different agroecological zones and two research institutes (Table 1).

Table 1: List of sampling locations

SC*	Sampling locations
01	Benin-Edo State
02	Kadaroko-Nassarawa State
03	Oye-Ekiti-Ekiti State
04	Ilorin-Kwara State
05	Nhst-0588
06	Makurdi-Benue State
07	Rumibekwe-River State
08	Ikom-Cross State
09	Oshogbo-Osun State
10	Ikwuano-Abia State
11	Elelenwo-Rivers State
12	Nagrab-00753
13	Ukwa-Abia State
14	Iberenta-Abia State

*SC: Sample code

DNA extraction

Extraction of DNA was done in the molecular laboratory of Michael Okpara University of Agriculture Umudike, Abia State, Nigeria. DNA extraction was done with a modified SDS extraction method by Dellaporta *et al.* (1983). Samples were prepared by using approximately

100g silica gel dried leaves tissue in an extraction tube. Two steel balls were added to the tube to enable grinding. The dried leaf tissues were ground into fine powder by vortexing for five minutes. About to 750µl of pre-heated extraction buffer was added. The tubes were mixed occasionally inverted and incubated at 65^0C for 20 min to homogenize the sample. The tubes were removed and allowed to cool for 2 min, and then 200 µl of ice-cold 5 M Potassium acetate was added to them and incubated on ice for 20 minutes to precipitate protein. 500 µl of chloroform Isoamyl alcohol (24:1) was added to each tube and mixed gently to further precipitate protein and lipids. It was then centrifuged at 111.8 g for 10 min, and then the supernatant was transferred into freshly labeled tubes. In the last stages to precipitate, the DNA was to add two-thirds of a volume of ice-cold isopropanol, mix gently, and rotate at -80 ^{0}C for fifteen minutes. After a 10-minute centrifugation at 111.8 g, the supernatant was poured off until the last drop. Washing the DNA pellet required 400 µl of 70% ethanol. Once the centrifugation was run for 10 minutes at 111.8 g, the supernatant was drained off to the last drop. To remove the ethanol smell, the DNA pellet was allowed to air dry. To re-suspend the DNA, 60 µl of ultra-pure water was gently mixed and added. Thereafter, 2 µl of RNase was incubated at 37 ^{0}C for 30- 40 minutes.

Amplification of ITS region

ITS sequences of rDNA were amplified using primers of White *et al*. (1990). ITS1 (Forward 5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (Reverse 5'-TCCTCCGCTTATTGATATGC-3') with the polymerase chain reaction (PCR) using the AccuPower HF PCR PreMix (Bioneer, Daejeon, South Korea) in 20 µl of total volume including H_2O deion (7µl), 2X PCR master mix solution of (1.0µl), 10pmol/µl of forward primer (1.0µl), 10pmol/µl of reverse primer (1.0µl) and 50ng/µl of DNA template (1.0µl). One round of amplification consisted of denaturation at 94°C for 5 min. It followed by 40 cycles of denaturation at 94°C for 1 min., and annealing at 49°C for 1 min and extension at 72°C for 1 min. with a final extension step of 72°C for 5 min. The PCR products were purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) before sequencing.

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems, using the manufacturers' manual. The sequencing kit used was a BigDye Terminator v3.1 cycle sequencing kit. Bio-Edit software and MEGA 6 were used for all bioinformatics.

Sequence alignment and phylogenetic analysis

T.cucumerina sequences in FASTA format were aligned and used in the analysis of bioinformatics of the fourteen ecotypes, the process of sequence analysis was advanced through pairwise alignment, construction of a distance matrix using Mega X. The sequences were compared to the NCBI database using Nucleotide Basic Local Alignment Search Tool (BLAST). The results of the distance matrix were sent to Excel 2007, which was finally analyzed using PAST 3.14 software for the construction of a phylogenetic tree showing the similarity and distance relationship between the fourteen ecotypes.

Results and Discussion

Figure 1 shows the electrophoresis results of the pure DNA extracted from fourteen ecotypes of the landraces of T. cucumerina.

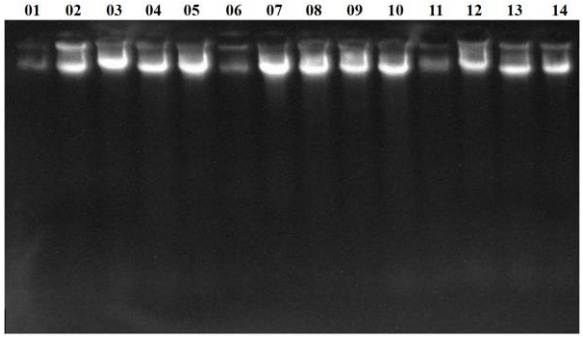

Fig. 1. Electrophoresis of the DNA extracted from of fourteen ecotypes of *T.cucumerina* in Nigeria: numbers indicate location of samples, which brought in the table1.

Xia *et al*. (2019) stated that modified SDS-based DNA extraction yielded high-grade DNA per this work. Trichosanthes cucumerina var anguina Nigeria landraces, GQ240882.1 and GQ059528.1, have 100% query coverage, 14,592 hit start, and end, 579,577 bp sequence

minimum and maximum, respectively. Ekiti State ecotype accession number GU059528.1 has 100% query coverage, 69,646 hits, and 578,577bp sequence minimum and maximum, while Ilorin, Kwara state's GU059528.1 *T. cucumerina var anguina* has the same. The NHST-0588 ecotype has a 99.48% query coverage with a sequence length of 577 bp (Table 2).

The number of base substitutions per site within sequences is shown. Analysis was done using maximum composite likelihood (Tamura *et al.*, 2004*)*. The analysis involved 14 nucleotide sequences. Codon positions included 1^{st}+ 2^{nd}+ 3^{rd}+ Noncoding. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

Genetic distance from ecotypes of closer locations is meager. Sánchez-Guillén *et al*. (2011) indicated the influence of location in genetic diversity studies; this might be responsible for the close relationship between members originating from close locations. The sequence alignment of the fourteen ecotypes is shown by inter transcribed spacer (ITS), which showed by query percentage coverage (Q%) in the table 2.

Table 2. Sequencing of fourteen accessions of *T.cucumerina* using ITS.

SL	AC	Descriptive	Seq	E	Q%	HS	HE	S max	S min
Benin-Edo State	GQ240882.1	*T. cucumerina var anguina*	2	0	100	14	592	579	577
Kadaroko-Nassarawa	GU059528.1	*T. cucumerina var anguina*	2	0	99.66	69	646	578	578
Oye-Ekiti-Ekiti State	GU059528.1	*T. cucumerina var anguina*	2	0	100	69	646	578	577
Ilorin-Kwara State	GU059528.1	*T. cucumerina var anguina*	2	0	100	69	645	578	577
Nhst-0588	GQ240883.1	*T. cucumerina var cucumerina*	2	0	99.48	14	590	579	577
Makurdi-Benue State	GU059528.1	*T. cucumerina var anguina*	2	0	100	646	695	577	576
Rumibekwe-Rivers State	GU059528.1	*T. cucumerina var anguina*	2	0	100	69	645	577	576
Ikom-Cross State	GU059528.1	*T. cucumerina var anguina*	2	0	100	69	645	576	573
Oshogbo-Osun State	GU059528.1	*T. cucumerina var anguina*	2	0	100	69	645	578	577
Ikwuano-Abia State	GU059528.1	*T. cucumerina var anguina*	2	0	100	69	645	577	577
Elelenwo-Rivers State	GU059528.1	*T. cucumerina var anguina*	2	0	100	69	645	578	570
Nagrab-00753	GU059528.1	*T. cucumerina var anguina*	2	0	100	69	645	578	577
Ukwa-Abia State	GU059528.1	*T. cucumerina var anguina*	2	0	100	69	645	580	577
Iberenta-Abia State	GU059528.1	*T. cucumerina var anguina*	2	0	100	69	645	577	573

***** SL: Sampling locations, AC: Accession number, Seq: Sequence, E: E-value, Q%: Query percentage coverage, HS: Hit start, HE: Hit end, S max: Sequence max, S min: Sequence min.

Phylogenetic assessment

The dendrogram was constructed using Neighbour-joining. The analysis divided the accessions into three major groups, with group two having the highest number of accessions together; this was followed by group Three, while group one had the lowest number of accessions group together. There was a close relationship between *T.cucumerina var anguina* collected from NAGRAB 00753 and Ukwa, Abia State. Benin and Oye-Ekiti are closely related to each other. Elelenwo, Ikom, Makurdi, Rumibekwe, Iberenta, and Ikwuano have a very close relationship with each other. Ilorin, Oshogbo, and Kadarko are related to each other according to the phylogenetic tree. NHST 0588 from the seed center in Ibadan is the only Trichosanthes cucumerina var cucumerina among the landraces sequence and blasted in NCBI websites. The similarity coefficient is 0.714.

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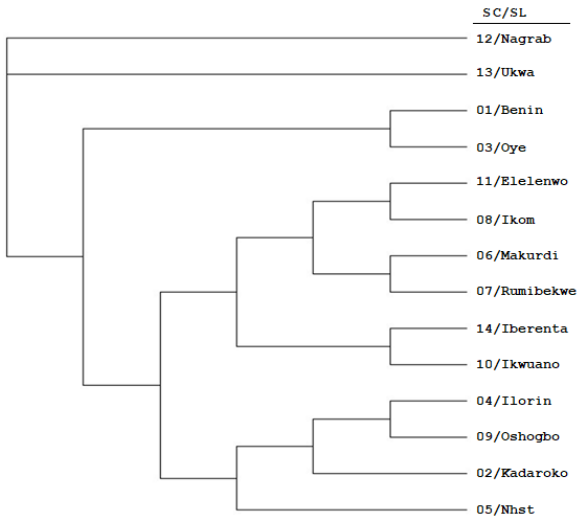

Fig. 2. Phylogenetic tree of ITS which characterized the fourteen ecotypes *T.cucumerina*: SC/SL: sample code or sampling locations

Hapsari *et al*. (2018) reported genetic variability and relationship of banana cultivars (Musa L.) from Indonesia using ITS. They observed that the DNA sequence length of 41 banana cultivars ranged from 631-651bp, which also indicates high variability with a conservation level of 62.79%. This work did not agree with this research on snake tomatoes. The work of Alagan *et al*. (2019), who reported the identification of Justicia gendarussa using an ITS, showed that a DNA sequence of 493bp with query coverage of 99% aligns with this work. The work of Ali *et al*. (2011), who reported genetic diversity among Indian populations of Cuscuta reflexa using ITS, indicated variability among the 30 species. It classified them according to region, which concurs with this research.

Awala *et al*. (2019) reported the landraces of *Lagenaria siceraria* in Nigeria as a result of its complexity using the rbcl marker. They observed that sequence results showed that the species studied belong to Lagenaria siceraria with 95-100% query cover sequence and 98-100% for identity sequences. This is in agreement with this work. The work of Girme *et al*. (2022) reported the identification of DNA barcodes for seven species of Momordica using a matK marker and observed that matK yielded distinct barcodes in *Momordica charantia* var *charantia*, *M. subangulata*. subsp. *Nigeria*, *M. cocohinchiinesis*, *M. balsamina*, *M. cymbalaia* and also in *Luffa acutangular*. The wild species of *M. dioica* and *M. sahyadrica* shared one barcode at 585bp, which agrees with this work. Vaez-Sarui *et al*. (2022) reported evaluating the genetic diversity of Cantaloupe landraces based on the internal transcriptional spacer regions. They observed that melon could not be separated based on geographical location which affirmed this work. Hidayat *et al*. (2021) reported phylogenetic characterization of Apple Cucumber from Indonesia and observed that apple cucumbers are grouped with melon (Cucumis melo).

In conclusion, this research investigated characterization of 14 ecotypes of Snake gourd landraces in Nigeria using ITS region. Among fourteen studied Nigeria ecotypes and landraces *T. cucumerina*, thirteen were *anguina* varieties, and the NHST-0583 from the seed center was the only *cucumerina* variety. Among fourteen studied Nigerian ecotypes and landraces of *T.cucumerina*, thirteen were *anguina* varieties, and the NHST-0583 from the seed center was the only *cucumerina variety*.

Disclaimer

The products used for this investigation are regularly and mostly used in our research area in Nigeria. There is undeniably no conflict of concern between the authors and growers of the products since we do not plan to use these products as an opportunity for litigation but for the progress of knowledge. Also, this research was not financed by the producing enterprise but by the personal efforts of the authors.

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Disclosure Statement

The authors have declared that no competing interests are in existence.

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