Dna Barcoding: Efficiency of Rbcl Gene for Sequence Characterisation of Cyperus Esculentus in Nigeria

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Research Article

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Abstract

Background

Plants have been classified based on morphological traits. However, there are biases associated with this classification. The limitation can be prevented by using DNA barcoding. Therefore, the efficiency of the Ribulose -bisphosphate carboxylase (rbcL) marker was investigated to identify Tiger Nut (*Cyperus esculentus*), which belongs to the family of Cyperaceae. In this study, *C. esculentus* samples were collected from two villages in northern Nigeria. For DNA barcoding, the rbcL region of *C. esculentus* samples were amplified, sequenced in both directions, and subsequently analysed. The amplified sequences' biological sequence homology, sequence divergence, and phylogenetic tree construction were studied using Basic Local Alignment Tool (BLAST), Codon Code Aligner and MEGA X respectively.

Results

A 100% identity of rbcL locus with several other Cyperus species was obtained from BLAST result. The region seems highly conserved among several Cyperus species as variation within this marker locus's sequence is minimal. However, a G/T variation at a nucleotide position could differentiate the tested samples from 40% of the other data bank species. The phylogenetic tree analysis clustered the 20 samples and the retrieved sequences based on the very few sequence variations without distinctly separating *C. esculentus* samples from other Cyperus species.

Conclusions

These findings showed that rbcL marker could only identify our samples up to genius level. Therefore, other DNA barcoding markers are needed to identify and characterise *C. esculentus* since the characterization based on rbcL gene sequences conducted in this work could not identify *C. esculentus* up to the species level.

Background Study

Tiger nuts (chufa flatsedge, yellow nutsedge) are edible tubers of *Cyperus esculentus*, an underutilised herbaceous perennial plant. It belongs to the Cyperaceae family, which produces rhizomes and spherical tubers (Fig. 1). It is widespread across much of the world (Sanchez-zapata *et al.*, 2012). Cyperaceae are ranked as the seventh-largest family in the angiosperms and classified as the third largest in the monocotyledons (Simpson et al., 2011). Tiger nut is also called tiger nutsedge, chufa sedge, yellow nutsedge, earth almond, yellow nutgrass, chufa, Zulu nut (Belewu and Belewu., 2007; Omode et al., 1995). Tiger nut is mainly cultivated in Africa and can also be found almost in all temperate, tropical, and subtropical regions of the world (Bazine and Arslanoglu, 2020; Govaerts, 2014). Cyperus esculentus is considered an underutilised crop and can be found as a weed, crop or wild (Ukwuru and Ogdobo 2011;

Govaerts et al., 2007; De Vries., 1991). Since ancient times, *C. esculentus* has been considered a foodstuff (Pascual et al., 2000). *Cyperus esculentus* is considered a native of the old world and an essential food in ancient Egypt (Negbi., 1992). About 6000 years ago, its dry tubers were found in predynastic tombs (Zohary., 1986). In other parts of the world, no records of this plant are documented (Zohary and Hopf., 1993).

Tiger nut is mainly consumed raw, especially as it is uniquely sweet. It can be processed into gluten-free flour, beverages, milk, edible oil, and beer. Its medicinal benefits include reduced risk of colon cancer, function as a heart stimulant and remedy for diarrhoea, and anti-inflammatory (Maduka and Ire, 2018; Achoribo and Ong, 2017; Adejuyitan, 2011; Adejuyitan et al., 2009). Tiger Nut milk has been reported to be more refreshing than other soft drinks and very healthy due to its contribution to the reduction of cholesterol (Belewu and Abodunrin, 2008). *C. esculentus* has carminative, diuretic, aphrodisiac, emmenagogue, stimulant and tonic effects (Chopra et al., 1986; Chevallier, 1996). It has been established in the protein standard proposed by the FAO|WHO that *C. esculentus* contain higher essential amino acids than the proposed standard (Bosch et al., 2005). Tiger nut is very nutritive and energetic for both young and old. Tiger nut flour is abundant in oil, oleic acid, fibre, carbohydrates, lipids (Muhammad et al., 2011), and some helpful mineral components, for example, iron and calcium, which are fundamental for body development and improvement (Oladele and Aina, 2007). Plant-based diets' nutritional profile and health benefits have recently gained increased awareness, especially the advantages of consuming fruits, vegetables, and other plant-based foods that can be consumed with minimal post-harvest processing. *Cyperus esculentus* L. (Tiger nut plant) is a relevant example of a plant with tremendous nutritional benefits and traditional medicinal uses. However, it is underutilised and has limited biological information on its uses. The health benefits and constraints to crop production coupled with its conventional medicine applications necessitate plant identity preservations to prevent the loss of cultural knowledge about such plants and misrepresentation, especially as *C. esculentus* is often confused with other *Cyperaceae* (Lauwers et al., 2020; Mezzasalma et al., 2017).

DNA barcoding is a new tool designed to identify species based on conserved sequences of nucleotide diversity. It is currently gaining popularity among plant scientists due to its simplicity and high accuracy in reviewing genetic diversity, species discrimination and phylogeny. Molecular barcoding is the best technique to screen for adulteration in medicinal plants (Sundari *et al*., 2019). DNA barcoding loci are important modern tools for unfolding the genetic relationships among plant species (Shawkat, 2019). DNA barcoding uses internationally agreed protocols and regions of DNA to create a global database of living organisms for species identification (Vere et al., 2015; Hollingsworth et al., 2011; Hebert *et al*., 2005). Identification of unknown plant samples, as well as evaluation, understanding, preservation, and utilization of biodiversity in a presented way, have been achieved step-by-step by studying and comparing novel plant barcode sequences with the sequences of the global DNA reference libraries (Kress et al., 2015). DNA barcoding approaches have been used widely for the identification of plant products ranging from tea (Stoeckle et al., 2011) to kitchens spices (De Mattia et al., 2011), berries (Jaakola et al., 2010), olive oil (Kumar et al., 2011) and medicinal plants (Asahina et al., 2010, Chen et al., 2010). Phylogenetic trees have been constructed in phylogenetic community ecology using genetic sequences generated from

DNA barcoding approaches (Kress et al., 2009; Kress et al., 2010). The Consortium for the Barcoding of Life (CBOL) plant working group (PWG) recommended that a standard region of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) and maturase K (matK) for the barcoding of all land plants (CBOL). Chloroplast DNA (cpDNA) are DNA barcodes often used in plants. rbcL gene is about 1400bp long, providing many characters for phylogenetic studies (Sundari *et al.*, 2019). In this study, a DNA barcoding approach was used to characterize 20 Tiger nuts samples. The sequences were compared with those of other Cyperus species stored in public databases to investigate the rbcL gene's efficiency in identifying *Cyperus esculentus*. The study will be helpful in the molecular identification and characterization of Tiger nuts in Nigeria which will provide beneficial information to the food industry and medical industry in Nigeria.

Methods

Plant material and DNA Extraction

Young leave samples of Tiger nut from 20 different plant stands were collected into a Ziploc bag containing silica gel at Ningi Local Government Area of Bauchi State, Nigeria. The samples were transported from Bauchi to IITA Ibadan, where the experiments were carried out. DNA was extracted from leaf samples using the modified CTAB method (Doyle and Doyle, 1990). The concentration and quality of the extracted DNA were determined using gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo scientific). The ratio of absorbance at 260 and 280nm was used to assess the purity of DNA. The isolated genomic DNA was stored at -20°C until further use.

Pcr Amplification And Sequencing

Amplification of part of the rbcL gene fragment was carried out using the primers H1f F:(5 -CCACAAACAGAGACTAAAGC-3), Fofana R: (5 -GTAAAATCAAGTCCACCGCG-3). PCR reaction mixture of 25uL comprised the following: 3ul of template DNA (50ng), 2.5ul of 10X NH4 buffer (PCR buffer), 2ul of 2.5mM dNTPs, 2ul of 50nM $MgCl_2$, 1ul DMSO, 1ul Forward primer, 1ul Reverse primer, and 0.1ul Taq polymerase (5U/ul). The total volume was made up to 25ul using 13.4ul of Nuclease-free water.

PCR-based amplification of barcoding regions was performed using Veriti 96-well thermal cycler (Applied Biosystems). The reaction was set up in the PCR thermocycler using the touchdown method. The PCR cycles consisted of an initial denaturation at 94^{o}C for 3min, 10cycles of denaturation (15 s at 94^{o}C), annealing (30 s at 65^{o}C), and extension (1.5 m at 72^{o}C), followed by another 30 cycles of denaturation (15 s at 94^{o}C), annealing (30 s at 55^{o}C), and extension (1.5 m at 72^{o}C), and a final extension (5 m at 72^{o}C). Before sequencing, PCR products were resolved on a 1.5% (w/v) agarose gel to ensure amplicons from the samples had just a single band.

Sequencing reactions were performed with the BigDye Terminator kit v3.1 (Applied Biosystems). Sanger sequencing was performed using a 3130XL genetic analyser from Applied Biosystems at the Bioscience Unit, International Institute of Tropical Agriculture (IITA), Ibadan. Purified PCR products were bidirectionally sequenced.

SEQUENCE EDITING AND MULTIPLE SEQUENCE ALIGNMENT

The raw trace files were retrieved and imported into the CodonCode Aligner version (9.0.1) for sequence editing and alignment. All raw sequences were end clipped to maximise error rates below 0.1. A consensus sequence for each sample was generated from a pairwise alignment of the forward and corresponding reverse sequences for downstream analysis. The resulting sequences of the 20 samples were aligned to the reference sequence retrieved from the National Centre for Biotechnology Information (NCBI) genebank (sequence ID: MG227263) using ClustalW of the CodonCode Aligner.

Dna Barcoding Identification

About 221 DNA barcoding sequences were retrived from GenBank, which comprise 86 *Cyperus* species out of about 700 currently recognised species of the genus *Cyperus*. It included at least two sequences from each of the Cyperus species. To avoid misidentification, we ensured sequence overlapping with those generated in this study. From the obtained results, nucleotide sequences of close relative *Cyperus esculentus* with high identity scores (95–100% and E-value of zero) were picked for classification purposes. The ClustalW program generated multiple alignments of all the sequences. The selected sequences were used to construct a phylogenetic tree using the maximum likelihood method on Molecular Evolutionary Genetic Analysis (MEGA X) software (Kumar et al., 2018).

The nucleotide sequence data were submitted to GenBank with an accession number of OM672242

Results

Good quality DNA was extracted from the tiger nut leaf samples with a yield ranging from 305.2 to 1639.4 ng/ul. DNA quality checked on 1% agarose gel is shown in Fig. 2. The *rbcL* loci were successfully amplified, and a distinct single band of the expected size (650bp) was obtained per sample. The purified PCR products of each sample produced high-quality sequences with strong chromatogram signals in all the reads. After end trimming, the sequence length per sample was 460bp for *rbcL*.

Analyses of the sequenced region using the *rbcL* barcode markers did not show any polymorphism among the 20 samples, which could be that the same cultivar is being planted across both villages where the samples were collected. However, the obtained sequences were compared with those of other Cyperus species retrieved from GenBank.

Multiple alignments of sequences newly obtained from *Cyperus esculentus* samples alongside sequences from various Cyperus species were used to construct a tree. The phylogenetic tree clustered the newly generated 20 sequences and the retrieved 221 sequences based on the very few sequence variations without distinctly separating *C. esculentus* samples from about 26 other Cyperus species out of 86 species analysed (Fig. 3).

Discussion

DNA extraction from *Cyperus esculentus* can be difficult due to large amounts of secondary metabolites, although the extraction protocol worked for all the samples with good concentration and purity. The sequence analysis showed that the *rbcL* region is conserved among the samples, suggesting a low level of variation within and among the 20 samples of *Cyperus esculentus* in the studied region. There have been reports of low genetic diversity in both qualitative and quantitative characters within and among populations of farmers' cultivated tiger nut genotypes (Asare *et al*., 2020; Horak et al., 1987). A likely reason for this low variability is several years of seed sorting for homogenous planting materials. The *rbcL* DNA Barcode region of each of the 20 samples has 100% identity with about 30% of *rbcL* sequences of other Cyperus species, which infers no identification at the species–level was successful, but only genus identification was successful. This observation correlated with the report by (Hollingsworth et al., 2009; Roy et al., 2010). Their findings reported the limitation in the individual utilisation of primer sets as they were unlikely suitable for some of the lineages of land plants. De Groot et al. (2011) explained that when all the blast has maximal percentage identity scores greater than 95% involving a single genus, identification at the genus level was considered successful. Identifying species was deemed successful only when the highest percentage identity scores greater than 95% included a single species.

The earlier report reveals that *rbcL* was unable to identify *Cyperus articulates* which is a related species of *Cyperus esculentus* (Mezzasalma et al., 2017). According to Chen et al. (2010), this may be due to the small number of sequences per species. It is believed that species-level identification can only sometimes be achieved using DNA barcodes because reference databases often lack species and haplotype diversity (Heckenhauer et al., 2017). Wattoo *et al*. (2016) explored a combination of rbcL + matK to effectively identify and discriminate three divergent plants at the species level. Also, the combination of rbcL, matK, and ITS2 has been used by Pathak et al (2018) to identify medicinal plants in the Bahrain kingdom.

A Phylogenetic tree is a diagram that estimate the relationships among taxa or sequences and their hypothetical common ancestor (Nei and Kumar. 2000; Felsenstein, 2004; Hall, 2011). Using sequence of interest as a query to do a BLAST is the most reliable way to identify sequences that are homologous to the sequence of interest (Altschul et al., 1997). The phylogenetic tree clustered the newly generated 20 sequences based on the very few sequence variations without distinctly separating *C. esculentus* samples from about 26 other Cyperus species out of 86 species analysed. Thus, this may be caused by an unequal rate of evolution.

Conclusions

This study indicates that the standard core barcode marker, rbcL, is not suitable for distinguishing *Cyperus esculentus* from other close relatives due to the low level of variation in the region. Therefore, we suggest the need for designing and optimizing species-specific rbcL markers, which may be an effective tool for barcoding various plant species. Also, to the best of our knowledge, this study proved to be the first attempt at generating DNA sequences from *rbcL* barcoding loci in *Cyperus esculentus* species cultivated in Nigeria. Furthermore, this study provides a DNA extraction protocol and PCR amplification method that will play an important role in the efficient isolation and amplification of genomic DNA from *Cyperus esculentus*. This study would further be helpful in studying taxonomy, the ecology, and morphology of *Cyperus esculentus* and related species. However, the development of new protocols and new primers would significantly revolutionize DNA barcoding by describing more detailed genome information from different species.

Abbreviations

rbcL- Ribulose -bisphosphate carboxylase, BLAST- Basic Local Alignment Search Tool, PCR- Polymerase Chain Reaction, MatK- Maturase K

Declarations

Ethics Approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of Data and Materials

The data that support this finding of this study is available on request from the corresponding author (belloridwanopeyemi@gmail.com)

competing interests

The authors declare no known competing financial interests or no personal relationships that could have appeared to influence the work reported in this paper.

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Authors' Contributions

ROB: Conceptualization, methodology, Software. **TES, DB, FMT:** methodology, Data curation. **ROB, DB:** writing- original draft preparation. **AOO:** Supervision **AOO, TES, DB, FMT:** Writing: Reviewing and Editing. All the authors read and approved the final version for submission.

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Figures

Figure 1

The Root of *Cyperus esculentus* with Seeds

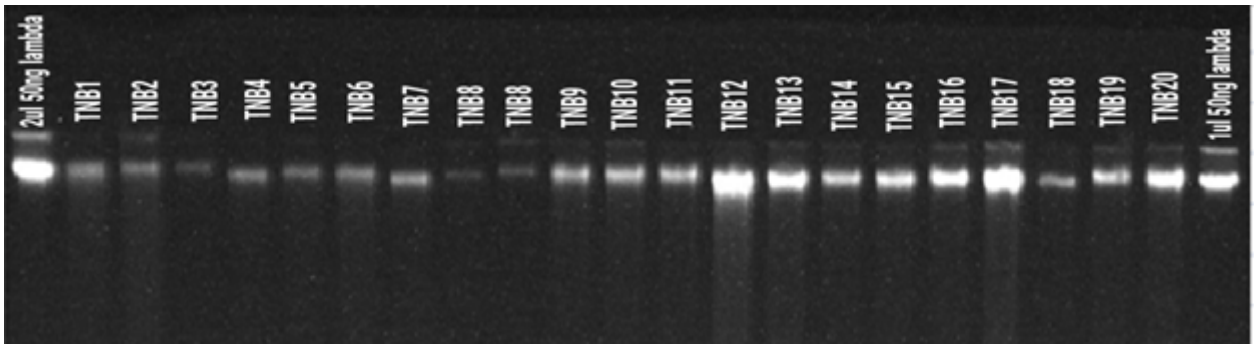

Figure 2

Agarose gel image showing the quality of genomic DNA extracted from *Cyperus esculentus*

Figure 3

Phylogenetic analysis of 241 partial *rbcL* sequences from 86 Cyperus species by maximum likelihood method.